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# THE FORMATION IN VITRO OF THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE WITH RADIOACTIVE IODINE AS INDICATOR\*

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The conversion of radioactive inorganic iodine to radiothyroxine and radiodiodotyrosine by excised surviving thyroid tissue is demonstrated here. Thyroid tissue removed from sheep, dogs, and rats was sliced and added to a bicarbonate-Ringer's solution containing approximately 0.1  $\gamma$  of  $I^{127}$  per cc. A tracer amount of  $I^{131}$  was added to this solution. The presence of newly formed radiothyroxine and radiodiodotyrosine was established by removal of radioactive contaminants with a dilution or "washing out" procedure during recrystallization of the radioactive compounds to constant specific activity.

*Thyroid Slices of Sheep, Dog, and Rat*—The formation of diiodotyrosine and thyroxine by thyroid tissue obtained from sheep, dog, and rat is shown in Table I. The thyroids of sheep and dog were sliced, whereas in the rat the gland was excised *in toto* and halved with a razor blade. At the end of 2 hours 31 to 37 per cent of the  $I^{131}$  contained in the Ringer's solution was converted to diiodotyrosine by sheep glands. In the same time excised dog thyroids showed a conversion of 21 to 24 per cent. A surprisingly large amount of  $I^{131}$  was found in the form of diiodotyrosine at the end of 3 hours in the bath containing rat thyroids: 60 to 71 per cent of the radioiodide was converted to diiodotyrosine in 3 hours by 320 to 360 mg. of rat thyroid tissue.

In 2 hours sheep thyroids converted 5 to 6 per cent of the Ringer's  $I^{131}$  to thyroxine. Somewhat less was found in the vessels containing 110 to 140 mg. of dog thyroid tissue for the same time. The most pronounced conversion of iodide to thyroxine occurred in the experiment with rat thyroid. The flasks in which 320 to 360 mg. of rat thyroid were incubated for 3 hours contained as much as 12 per cent of the  $I^{131}$  added to the Ringer's medium in the form of thyroxine.

*Identification of Newly Formed Diiodotyrosine and Thyroxine*—Although only two iodine-containing compounds, namely diiodotyrosine and thyroxine, have so far been identified in animal tissues, nevertheless the possible presence of other iodinated compounds in animal tissues must be

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# THYROXINE AND DIODOTYROSINE FORMATION

considered. Two such compounds are particularly worthy of mention. Diiodothyronine has not been isolated from animal tissues, but if present would be found mainly in the diiodotyrosine fraction in the procedure used in the present investigation (1). Monoiodotyrosine has recently been isolated by Ludwig and von Mutzenbecher from iodinated casein (2); if present in the animal body, this substance would probably follow the diiodotyrosine fraction. In view of these considerations, the actual presence of *radiothyroxine* and *radiodiiodotyrosine* was established as follows: 0.5 gm. of sheep thyroid slices was incubated for 2.5 hours in 5 cc. of a bicarbonate-Ringer's solution containing tracer amounts of radioiodide. After the addition of 6 cc. of 3.5 N NaOH, the mixture was hydrolyzed on

TABLE I  
*Thyroxine and Diiodotyrosine Formation by Thyroid Slices in Vitro*

Experiment No.	Animal	Thyroid tissue added to Ringer's solution*	Volume of Ringer's solution	Incubation period	Per cent of Ringer's $I^{131}$ recovered as		
					Thyroxine	Diiodotyrosine	Inorganic
55	Sheep	mg.	cc.	hrs.			
56	"	126	2	2	5.0		
59	"	112	2	2	5.1	33.7	61.3
60	"	120	2	2	5.7	37.1	57.8
57	Dog	121	2	2	4.9	33.3	61.0
58	"	114	2	2	3.4	30.8	64.3
61	"	137	2	2	3.6	23.7	72.9
62	"	138	2	2	3.0	20.8	75.6
80	Rat†	133	2	2	3.0	21.3	75.7
89	"	316	3	3	10.3	21.9	75.1
90	"	321	3	3	8.4	71.4	18.3
		362	3	3	11.7	60.0	31.6
						70.8	17.5

\* The bicarbonate-Ringer's solution used contained approximately 0.1  $\mu$  of  $I^{131}$ .  
† Whole glands were halved; glands pooled from ten rats in each experiment

a steam bath for 8 hours. After cooling, the hydrolysate was acidified with concentrated HCl. 2 cc. of 0.02 M KI were added as carrier, and the  $I_2$  produced by oxidizing with 1 cc. of 0.02 M  $KIO_3$  extracted with  $CCl_4$ . The aqueous layer containing the organically bound iodine was made to contain 20 per cent technical NaOH by adding 6 gm. of the solid material. A single extraction with 30 cc. of butyl alcohol was then made, and the butyl alcohol fraction evaporated to dryness under reduced pressure. 20 mg. of non-radioactive crystalline thyroxine were added to the butyl alcohol residue, and the mixture taken up in hot 0.10 N  $K_2CO_3$ . The thyroxine was recrystallized by way of the potassium salt, as described by von Mutzenbecher (3). The alkaline aqueous fraction (aqueous layer

after the first butyl alcohol extraction) was acidified to pH 3.5 to 4.0 with HCl. Six extractions with 20 cc. portions of butyl alcohol were made. The butyl alcohol layers were combined and then evaporated to dryness under reduced pressure. 20 mg. of non-radioactive crystalline diiodotyrosine were added to the residue (obtained after the evaporation of the butyl alcohol) and the mixture taken up in hot 70 per cent ethyl alcohol. The thyroxine and the diiodotyrosine were each recrystallized five times further, the thyroxine as the potassium salt and the diiodotyrosine from 70 per cent ethyl alcohol. In each recrystallization of thyroxine 5 mg. of non-radioactive diiodotyrosine were added in order to "wash out" any contaminating radiodiiodotyrosine by dilution. In the case of the diiodotyrosine recrystallizations, 5 mg. of non-radioactive thyroxine were added to "wash out" contaminating radiothyroxine. After each recrystallization the respective specific activities of the thyroxine and diiodotyrosine

TABLE II

*Recrystallization of Thyroxine and Diiodotyrosine to Constant Specific Activity*

The radioactivity is measured in counts per minute.

Recrystallization	Thyroxine			Diiodotyrosine		
	Colorimeter reading	Radioactivity	Specific activity	Colorimeter reading	Radioactivity	Specific activity
1st	64	100	1.56	264	2800	10.6
2nd	352	450	1.23	157	2200	14.0
3rd	390	522	1.34	410	5660	13.8
4th	423	530	1.25	362	5140	14.2
5th	673	875	1.30	125	1730	13.8

were determined and expressed in terms of radioactivity per unit reading of a Klett-Summerson photoelectric colorimeter (Table II).

The Kendall-Osterberg  $\text{HNO}_2$  color reaction (4) was found to give a means of determining quantitatively the pure thyroxine and diiodotyrosine obtained by recrystallization. The basis for this method is the color reaction obtained when an alcoholic solution of an *o*-hydroxydiiodo derivative of benzene is made alkaline with  $\text{NH}_3$  after treatment with  $\text{HNO}_2$ .

Quantitative results with the Kendall-Osterberg reaction were obtained when care was taken to use only pure thyroxine and diiodotyrosine. Impurities such as sulfate, acetate, etc., were found to cause the appearance of an orange color of variable intensity. For this reason the thyroxine was carefully precipitated as the potassium salt from a carbonate solution and the diiodotyrosine was precipitated from 70 per cent ethyl alcohol. After each recrystallization 2 to 3 mg. of the pure crystalline compound were dissolved in 5 cc. of 95 per cent ethyl alcohol in a small test-tube. To this

solution 0.2 cc. of 6 N HCl was added and this was followed by the addition of 0.5 cc. of a freshly prepared 1 per cent  $\text{NaNO}_2$  solution. A yellow color appeared that deepened by heating the solution just to the boiling point in a hot water bath. The solution was then cooled and 0.5 cc. of concentrated  $\text{NH}_3$  added. The resultant color was a deep pink. Variable aliquots of this solution were chosen according to the color intensity and amount of radioactivity present. To determine the color intensity the aliquot was transferred to a colorimeter tube and diluted accurately to the 5 cc. mark with 95 per cent ethyl alcohol. The color was found to remain constant with time and no tests were carried out at temperatures other than those of the laboratory. Colorimeter readings were found to be directly proportional to the amount of pure thyroxine or pure diiodotyrosine present.

Determinations of the radioactivity of the crystalline diiodotyrosine and thyroxine were made as described below by transferring an aliquot directly to a copper-foil disk and counting its activity on the Geiger-Müller counter. The radioactivity of all samples was counted at the same time, thus eliminating the necessity for corrections of radioactive decay. Specific activity was then expressed by dividing the counts of radioactivity per minute by the colorimeter reading obtained for the aliquot. Each determination was made in duplicate and the average values are recorded in Table II.

The results recorded in Table II show that a constant amount of radioactivity remains associated with each compound despite repeated recrystallizations. The specific activities<sup>1</sup> for both thyroxine and diiodotyrosine do not change significantly after the second recrystallization. This should be expected to occur only in a case in which the substance giving the radioactivity was identical with the material that underwent repeated recrystallizations. If two substances were involved, one being adsorbed on the other, it is hardly likely that a constant specific activity could be maintained by repeated recrystallizations that were accompanied by the "washing out" procedure. *When radioiodide or radiodiiodotyrosine was added to non-radioactive crystalline thyroxine, only two recrystallizations of the latter were found necessary to remove the radioactive substances from the thyroxine completely.* A similar result was obtained when radioiodide or radiothyroxine was added to non-radioactive diiodotyrosine and recrystallizations of the latter carried out; no radioactivity was found associated with the crystalline diiodotyrosine after its second recrystallization. These results strongly suggest the presence of newly formed thyroxine and diiodo-

<sup>1</sup> Since the colorimeter reading is proportional to the amount of thyroxine or diiodotyrosine, specific activity was measured by the ratio of the radioactivity to the colorimeter reading.

tyrosine, for the  $I^{131}$  of the thyroxine-like fraction cannot be due to contamination with either radiodiiodotyrosine or radioiodide, and the  $I^{131}$  of the diiodotyrosine-like fraction cannot be ascribed to contamination with radiothyroxine or radioiodide.

*Thyroxine and Diiodotyrosine Formation As a Function of Time*—In the experiments recorded in Table III, slices were prepared from the thyroid glands of two dogs. An amount of slices varying from 48 to 70 mg. was incubated in 2 cc. of a bicarbonate-Ringer's solution to which had been added a tracer amount of  $I^{131}$ . Zero intervals refer to experiments in which thyroid slices were hydrolyzed *immediately after* the addition of the radioiodide. The reliability of the fractionation procedure is shown by the

TABLE III

*Effect of Time on Amount of Ringer's  $I^{131}$  Converted to Thyroxine and Diiodotyrosine by Slices of Dog Thyroid Gland*

Duration of incubation	Thyroid tissue added*	Per cent of Ringer's $I^{131}$ recovered as		
		Thyroxine	Diiodotyrosine	Inorganic
<i>hrs.</i>	<i>mg.</i>			
0	66	0.7	1.4	97.7
0	61	0.6	0.8	98.9
1.5	51	1.7	6.9	91.5
1.5	64	2.0	6.7	91.5
3.0	57	3.8	21.3	75.2
3.0	48	4.3	21.2	74.5
4.5	70	4.8	30.6	64.6
4.5	54	4.6	29.9	65.6

\* Each flask contained 2.0 cc. of bicarbonate-Ringer's solution.

distribution of the radioiodide among the three fractions at this time interval.

A sharp increase in the radiodiiodotyrosine formed was observed between 1.5 and 3 hours. Between these two intervals, the  $I^{131}$  content of the diiodotyrosine rose from 7 to 21 per cent. The radiothyroxine during this time rose from about 2 to 4 per cent. During the next 1.5 hours (*i.e.* from 3 to 4.5 hours, in Table III) the increased incorporation of the  $I^{131}$  into diiodotyrosine and thyroxine was less pronounced than during the preceding 1.5 hours.

*Rôle of Tissue Organization in Formation of Radiodiiodotyrosine and Radiothyroxine*—In Table IV, the radiothyroxine and radiodiiodotyrosine formed in the presence of the following thyroid preparations were compared: sliced, minced, mortar-ground, and homogenized. In the first four experiments carried out with sheep thyroids, slices were prepared from

several glands and these slices pooled. In Experiment 1, slices were chosen at random and incubated in the bicarbonate-Ringer's solution containing  $I^{131}$ . In Experiments 2 to 4, randomly chosen slices were either minced with a razor blade on a glass plate or ground in an agate mortar, or homogenized. In Experiment 5 the thyroid glands of ten rats were homogenized in an all-glass apparatus described by Potter and Elvehjem (5), and treated in a manner similar to that of Experiment 4.

A progressive decrease in the radiothyroxine and radiodiiodotyrosine formed was observed in the order in which the preparations are listed. Thus from three-fifths to two-thirds of the  $I^{131}$  organically bound by slices was so bound in the case of minced tissue. A further decrease in the con-

TABLE IV

*Effect of Tissue Intactness on In Vitro Formation of Thyroxine and Diiodotyrosine*

Experiment No.	Animal	Tissue added*	Treatment of tissue	Time incubated	Per cent of Ringer's $I^{131}$ recovered as		
					Thyroxine	Diiodotyrosine	Inorganic
		mg.		hrs.			
1	Sheep	305	Slices	2	7.3	57.3	35.4
1	"	303	"	2	6.8	50.9	42.3
2	"	300	Minced	0	0.72	1.07	98.7
2	"	300	"	2	4.1	33.7	62.2
2	"	300	"	2	3.9	32.2	63.9
3	"	300	Mortar-ground (agate)	0	0.54	0.63	98.8
3	"	300	" "	2	2.9	21.0	75.9
3	"	300	" "	2	2.9	25.6	71.6
4	"	300	Homogenized in stainless steel	2	0.73	3.8	96.0
4	"	300	" " " "	2	0.79	0.61	98.5
5	Rat	331	" " glass	3	1.1	5.5	93.4

\* Each flask contained 3.0 cc. of bicarbonate-Ringer's solution.

version of  $I^{131}$  to the two organic forms was noted in the sheep thyroid tissue ground in a mortar. No formation of radiothyroxine was observed in homogenized sheep or rat thyroid tissue, and very small amounts of  $I^{131}$  were converted to radiodiiodotyrosine by thyroid tissue so treated.

*Comment*—Although the specific activities of the iodine compounds in the Ringer's solution and in the tissue were not measured and hence the actual amounts of newly formed diiodotyrosine and thyroxine cannot be calculated, it is nevertheless possible to give minimum values based on the known amounts of  $I^{127}$  added to the bicarbonate-Ringer's solution. Approximately 0.1  $\gamma$  of  $I^{127}$  as iodide per cc. is contained in the bicarbonate-Ringer's solution. This means that in the rat data shown in Table I approximately 0.3  $\gamma$  of  $I^{127}$  is labeled. Therefore at the end of 3 hours

approximately 10 per cent of 0.3 or 0.03  $\gamma$  of  $I^{127}$  in the Ringer's solution has been incorporated into thyroxine. By a similar calculation it can be shown that 70 per cent of 0.3  $\gamma$  or 0.2  $\gamma$  of  $I^{127}$  has been incorporated into diiodotyrosine. These are minimum values; if the iodine of the tissue (iodide or organically bound) contributes to the amounts labeled, then the specific activities of these compounds would be lowered, and the values shown in Table I would then represent larger absolute amounts of thyroxine and diiodotyrosine.

It is not possible at present to make a distinction as to whether iodinated molecules such as diiodotyrosine and thyroxine, or whether uniodinated molecules such as tyrosine and thyronine, react with the labeled iodide added to the bath. It is important to note, however, that no  $I^{131}$  was incorporated into thyroxine or into diiodotyrosine when 50 mg. of desiccated thyroid tissue were incubated for 4 hours in 2 cc. of bicarbonate-Ringer's solution under conditions identical with those described above for the slice. Nor was the incorporation observed when the organization of the slice was disrupted by homogenization (Table IV).

#### EXPERIMENTAL

The sheep from which thyroid glands were obtained weighed approximately 30 kilos. The glands were removed at the abattoir soon after the animals were killed, immediately packed in ice, and brought to the laboratory. After the glands were dissected free from extraneous tissue, they were sliced with a razor blade and the slices transferred to a Petri dish containing a bicarbonate-Ringer's solution. Slices were then carefully selected for uniformity and thickness (approximately 0.2 mm.), blotted on filter paper, weighed quickly, and transferred to a 25 cc. Erlenmeyer flask containing a bicarbonate-Ringer's solution made up with reagent grade material. The flasks were then placed in a constant temperature water bath maintained at 38°. Dogs weighing approximately 10 kilos were anesthetized and their thyroids removed. The slicing was done in a manner similar to that used for the sheep, although smaller amounts of tissue were used in each flask. In the experiments in which rat thyroids were used, animals weighing 180 to 220 gm. were chosen. The rats were sacrificed by a blow on the back of the neck and their thyroids rapidly removed and halved with a razor blade. Further treatment was the same as that employed for the thyroid slices of dog and sheep.

The bicarbonate-Ringer's solution was prepared according to Krebs and Henseleit (6). From  $10^5$  to  $3 \times 10^5$  counts per minute of  $I^{131}$  (as measured with the Geiger-Müller counter) were present as iodide in the Ringer's solution of each flask. The preparation of the radioiodine has been described elsewhere (7). The  $I^{127}$  present in the bicarbonate-Ringer's solu-

tion was mainly that due to the amount contained in the NaCl. The radioiodide was added in 0.1 cc. of a solution of isotonic NaCl. The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$ , and the atmosphere above the solution and slices was displaced with this gas mixture immediately before the flask was placed in the constant temperature water bath and again after each hour during the incubation. The pH of the solution was adjusted to 7.4 to 7.5 just before the addition of the slices. Measurements were made with either a quinhydrone electrode or a glass electrode of the Beckman type.

After completion of the period of incubation, the tissue was hydrolyzed with 2 N NaOH for 8 hours on the steam bath. The fractionation procedure has been previously described (8). The modifications introduced were such as to restrict the volumes worked with in order that the tedious  $\text{CrO}_3\text{-H}_2\text{SO}_4$  ashing previously used might be eliminated. Thus the thyroxine, diiodotyrosine, and inorganic iodine fractions were made up to volume after separation, and aliquots were pipetted directly on a  $4 \times 6$  cm. copper-foil disk on the bottom of which lay a strip of lens paper. The aliquots were chosen so that each contained approximately the same amount of radioactivity. In order to keep self-absorption of the radioactivity the same in all determinations, equal volumes of an  $\text{Na}_2\text{SO}_4$  solution were evaporated on each copper dish by heating the latter over a hot-plate. In this way each sample contained the same mass of solid material after evaporation to dryness. The samples were covered with a piece of Scotch tape and wrapped around a thin wall Geiger-Müller counter to determine their radioactivity. Each of the fractions was determined separately. The completeness of the recovery is shown by the sum of the values of the three fractions.

#### SUMMARY

1. The conversion of iodide to thyroxine and diiodotyrosine by surviving slices of thyroid glands obtained from sheep, dog, and rat is demonstrated.
2. Evidence for the presence of newly formed thyroxine and diiodotyrosine was presented by the isolation of each as a pure substance from a mixture of its radioactive and non-radioactive forms and by repeated recrystallization of the mixture of both forms of each to constant specific activity. The radiothyroxine was washed free of contaminating radio-diiodotyrosine by the addition of the latter in its non-radioactive form during the recrystallizations of the thyroxine. Radiodiiodotyrosine was freed from contaminating radiothyroxine in a similar manner.
3. As much as 12 per cent of the added  $\text{I}^{131}$  was incorporated into thyroxine and as much as 70 per cent into diiodotyrosine when 300 to 350 mg.

of rat thyroid glands were incubated in a bicarbonate-Ringer's solution for 3 hours. In slices prepared from the thyroids of dog and sheep, as much as 4 and 6 per cent of the added  $I^{131}$  was converted to thyroxine respectively, and as much as 21 and 37 per cent was incorporated into diiodotyrosine.

4. The *in vitro* incorporation of  $I^{131}$  into thyroxine and diiodotyrosine by thyroid glands failed to occur when their organization was disrupted by homogenization.

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# ACETOIN: POLAROGRAPHIC DETERMINATION AND DISAPPEARANCE FROM THE BLOOD AFTER ADMINISTRATION

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Acetoin (acetylmethylcarbinol) has been found in minute amounts in normal human urine (1) and in the blood of the cow, pig, sheep, and horse (2, 3). Westerfeld, Stotz, and Berg (4) have reported that in dogs the rate of disappearance of alcohol from the blood was increased by the administration of pyruvate and that of pyruvate by the administration of alcohol. They interpret their results as indicating that acetaldehyde, produced by a primary oxidation of alcohol, may be condensed with pyruvate to form acetoin. They suggest that this reaction may furnish an explanation for a relationship between the metabolism of alcohol and of carbohydrate. Support is given to their suggestion by the fact that pyruvic acid is a product of the normal metabolism of dextrose and that the condensation of acetaldehyde and pyruvic acid to form acetoin is known to occur in the metabolic activity of yeast and bacteria. Green, Westerfeld, Vennesland, and Knox (5) have demonstrated this condensation *in vitro* with enzymes from tissues of various animals.

Westerfeld, Stotz, and Berg did not test their interpretation of alcohol metabolism by making determinations for acetoin in the blood of the dogs to which they gave alcohol and pyruvate. The investigation reported here is preliminary to such determination (6). The disappearance of acetoin from the blood following its administration was studied, since the possibility exists that it may be so rapid as to prevent the accumulation of appreciable amounts of acetoin.

## *Polarographic Determination of Acetoin*

Previous procedures for determining acetoin have consisted in the formation and gravimetric determination of nickel dimethylglyoxime by the method of Lemoigne (7). The weight of the precipitate is less than twice that of the acetoin from which it is formed; therefore, for reasonable accuracy, at least 2 mg. of acetoin must be present in the material to be analyzed. The estimation of acetoin in low concentrations in blood would require such large samples as to be impracticable for repeated determinations on laboratory animals. The analytical procedure based on nickel dimethylglyoxime is time-consuming. The polarographic method, to be

described here, is equally specific and quantitative; with it, acetoin in concentrations as low as 0.5 mg. per cent can be determined in 2 cc. of blood and the analysis completed in 1 hour.

The method in principle follows that of Lemoigne in the formation of diacetyl from acetoin and the separation by distillation. The diacetyl is then determined polarographically. Lemoigne oxidized the acetoin with small amounts of ferric chloride; van Niel (8) found that this procedure did not give constant yields of diacetyl and modified it by using a large excess of ferric chloride and distilling slowly. Stahly and Werkman (9) were unable to obtain consistent results even with the modification of van Niel. It was found in the present investigation that these discrepancies may result from carrying out the distillation before the acetoin is completely oxidized and that they can be obviated by heating the solution containing the acetoin and ferric chloride in a hot water bath for 30 minutes before distillation.

The procedure used here in obtaining the diacetyl is as follows: A 1:10 tungstic acid, protein-free filtrate is prepared from 2 cc. of blood by the Folin-Wu method. 10 cc. of the filtrate are placed in a 125 cc. distillation flask and 10 cc. of 50 per cent solution of ferric chloride added and an ebullition tube inserted. The flask is connected to a small vertical condenser, the lower end of which extends into a receiving tube marked at 10 cc. The distillation flask is immersed in a hot water bath for 30 minutes; the bath is then removed and the flask is heated directly with the flame from a micro burner. Distillation is continued until exactly 10 cc. are collected. A few cc. of the distillate are then taken for the polarographic determination of diacetyl.

Winkel and Proske (10), Adkins and Cox (11), and Tachi (12) have shown that diacetyl gives a reduction curve on the polarograph. The reduction potential and diffusion current are influenced by the pH of the solution and the nature of the supporting electrolyte. In the present investigation, sodium sulfite was used as the supporting electrolyte. It proved especially satisfactory for the following reasons: It gave a large diffusion current for diacetyl at a half wave potential of  $-0.86$  volt; the reduction potential of sodium occurs at a much higher negative potential and thus gives no interference; and the sulfite obviates the necessity of bubbling nitrogen through the solution to remove oxygen.

Kolthoff and Lingane ((13) pp. 59, 346) have confirmed the equation of Ilkovic (14) for inorganic ions and uncharged molecules but have shown that it does not apply to the reduction of all organic compounds. Therefore, preliminary to the determination of unknown quantities of acetoin by the method described here, measurements were made with a series of solutions of known concentrations, corrections were made for the residual

current ((13) p. 55), and a curve was plotted for diffusion current in relation to concentration. This curve, shown as Fig. 1, indicates that the relation between diffusion current and concentration was not linear. Fig. 1 was used as a reference curve for the estimation of unknown solutions of diacetyl.

For the determination with the polarograph, a cell of about 5 cc. capacity was used and determinations made on 2 to 3 cc. of distillate to which an excess of sodium sulfite crystals was added. If the concentration of diacetyl was so high that the diffusion current fell beyond the range of the reference curve, an appropriate dilution of another portion of the distillate was made and the determination repeated. Each mg. of diacetyl corresponds to 1.023 mg. of acetoin.

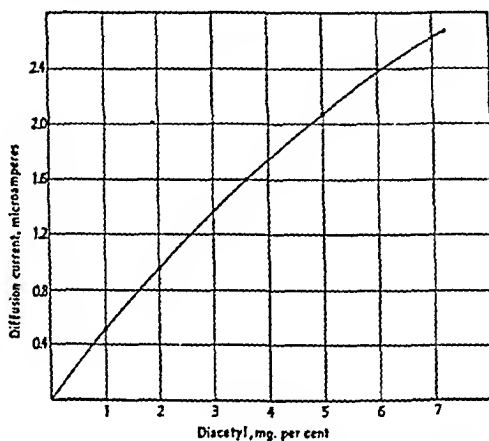


FIG. 1. Reference curve for concentrations of diacetyl

A series of determinations was made on known solutions of acetoin in water, blood, and urine; the results are given in Table I. Determinations were also made on the blood and urine used to assure initial freedom from acetoin. The acetoin was prepared by reducing diacetyl with zinc and sulfuric acid and was separated and purified by continuous ether extraction and fractional distillation (15). The purity was confirmed by the refractive index. With 1 mg. per cent of acetoin the maximum errors in single determinations on water, blood, and urine were  $\pm 0.2$  mg. per cent; with 10 mg. per cent of acetoin, the maximum errors were  $+0.2$  and  $-0.5$  mg. per cent on water,  $+0.2$  and  $-0.4$  mg. per cent on blood, and  $0.0$  and  $-0.5$  mg. per cent on urine; with 75 mg. per cent the maximum errors were  $+1.1$  and  $-1.2$  mg. per cent on water,  $+0.6$  and  $-1.0$  mg. per cent on

TABLE I

*Determination of Acetoin in Water, Blood, and Urine*

The values are given in mg. per cent.

Water			Blood			Urine		
Known	Found	Error	Known	Found	Error	Known	Found	Error
1.0	1.1	+0.1	1.0	1.2	+0.2	1.0	0.9	-0.1
	0.8	-0.2		0.8	-0.2		1.1	+0.1
	1.2	+0.2		0.9	-0.1		1.2	+0.2
	0.9	-0.1		1.1	+0.1		0.8	-0.2
	1.1	+0.1		1.0	0.0		1.0	0.0
	0.9	-0.1		0.8*	-0.2			
	1.1	+0.1		1.2*	+0.2			
	0.8	-0.2		1.0*	0.0			
	0.9	-0.1		1.1	+0.1			
	1.1	+0.1		0.8	-0.2			
Averages.....	0.99			0.99			1.0	
10.0	9.7	-0.3	10.0	9.6	-0.4	10.0	9.7	-0.3
	10.0	0.0		9.9	-0.1		10.0	0.0
	10.1	+0.1		9.7	-0.3		9.6	-0.4
	9.6	-0.4		10.0	0.0		9.9	-0.1
	9.5	-0.5		9.8	-0.2		9.5	-0.5
	9.8	-0.2		10.1	+0.1			
	9.7	-0.3		9.6	-0.4			
	10.2	+0.2		9.9	-0.1			
	9.9	-0.1		9.6	-0.4			
	10.1	+0.1		10.2	+0.2			
Averages.....	9.86			9.84			9.74	
75.0	74.2	-0.8	75.0	74.0	-1.0	75.0	74.1	-0.9
	75.0	0.0		74.3	-0.7		74.6	-0.4
	74.1	-0.9		74.3	-0.7		74.3	-0.7
	73.8	-1.2		74.1	-0.9		74.9	-0.1
	74.5	-0.5		75.6	+0.6		74.2	-0.8
	76.1	+1.1		74.2*	-0.8			
	75.8	+0.8		74.1*	-0.9			
	74.3	-0.7		75.3*	+0.3			
	74.2	-0.8		74.6	-0.4			
	75.1	+0.1		74.0	-1.0			
Averages.....	74.71			74.45			74.42	

\* Blood also containing 225 mg. per cent of ethyl alcohol.

blood, and -0.9 mg. per cent on urine. The addition of 225 mg. per cent of alcohol to the blood containing acetoin did not affect the accuracy of the determination of the acetoin. The increase in error with increase in

concentration of acetoin is due probably to greater loss of diacetyl during the distillation of the more concentrated solutions.

### *Rate of Disappearance of Acetoin from Blood*

Acetoin was given to six dogs in amounts of 1 or 2 gm. per kilo of body weight. The concentration of acetoin in the blood was determined at hourly intervals. The curves for concentration in relation to time were virtually identical for each animal given the same dose. The values obtained are shown in Fig. 2. The rate of disappearance of acetoin was not uniform but fell progressively with decreasing concentrations. When 1 gm. of acetoin was given, the concentration in the blood 1 hour later was 64 to 71 mg. per cent; this low concentration suggests that as with alcohol the acetoin diffuses into the intercellular as well as extracellular fluid of the body. In the next four hourly determinations the concentration of acetoin in the blood fell by 26, 22, 12, and 5 mg. per cent. Thereafter the acetoin disappeared at a very slow rate; so that at 9 hours after administration, 1 mg. per cent was still in the blood. Control samples of blood taken before injection of the acetoin showed none in the blood.

When 2 gm. per kilo of acetoin were given, the concentration at the end of 1 hour was 193 to 205 mg. per cent as compared to 64 to 71 mg. per cent after half the dose; the possibility exists that with the larger dose the distribution throughout the body was not complete at 1 hour and that part of the decrease in the concentration in the blood during the next 2 or 3 hours may have been due to withdrawal of acetoin by the tissues (16). Therefore, the possibility cannot be excluded by the experiments reported here that at the higher concentrations the disappearance from the blood, other than by distribution, may be at a uniform rate. Completion of distribution would unquestionably be reached with the larger dose in less than 4 hours and by the smaller in less than 2 hours; the distribution of ethyl alcohol (with similar solubility in water but less molecular weight) is reached in much shorter times. The progressive decrease in hourly disappearance of acetoin beyond these times is unquestionably valid. Even with the period of very slow disappearance of acetoin at low concentrations excluded, the general rate of disappearance is no more rapid than that of alcohol; after 1 and 2 gm. per kilo are given to a dog, the alcohol disappears from the blood in about 4 and 7 hours.

### *Acetoin in Urine*

The high boiling point of acetoin (148°) would prevent more than a trace of this substance appearing in the expired air but it has been shown by Neuberg and Gottschalk (17) that it may appear in the urine. In one experiment in which 2 gm. per kilo of acetoin were given to a dog, the bladder urine was withdrawn by catheterization each hour and the concen-

tration of acetoin determined. The curve obtained from these concentrations is shown in Fig. 2. The total loss of acetoin in the urine up to the time when the concentration of acetoin had fallen to 3.8 mg.

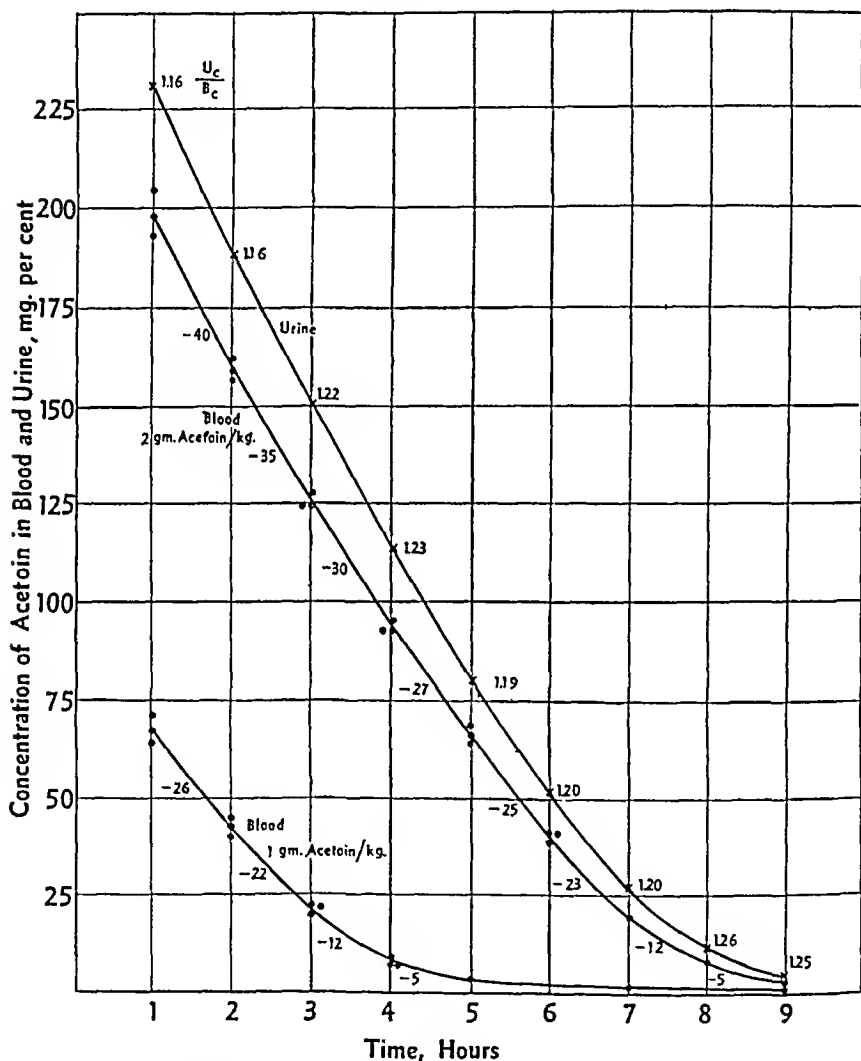


FIG. 2. Concentration of acetoin in the blood and urine following administration of 1 and 2 gm. per kilo to dogs.

per cent was 1.2 per cent of that administered. The concentration ( $c$ ) of acetoin in the urine ( $U$ ) was higher than in the blood ( $B$ ) and at a ratio ( $U_c/B_c$ , Fig. 2) which averaged 1:1.2. As with alcohol (18, 19), it would

appear probable that acetoin passes through the kidneys by diffusion and that the differences in concentration are indicative only of differences of solubility in blood and urine (19), and consequently may vary slightly with the amount of salts dissolved in the urine.

#### SUMMARY

1. A rapid and accurate polarographic method is described for the determination of acetoin in blood and urine.

2. The rate of disappearance of acetoin from blood was determined after the administration of 1 and 2 gm. per kilo to dogs. The rate, within the range studied, decreases with decreasing concentration. For the doses given, acetoin disappears from the blood no more rapidly than does ethyl alcohol.

3. Acetoin appears in the urine in a concentration approximately 1.2 of that in the blood. The percentage loss in the urine is small.

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# THE IRON CONTENT OF CRYSTALLINE HUMAN HEMOGLOBIN

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The determination of human hemoglobin has depended heretofore on assumed constants obtained from data on hemoglobin of other species. Peters and Van Slyke (1) have stressed the unreliability of these assumed values, which are 1.34 cc. of oxygen capacity per gm. or 0.335 per cent iron. The only recent work on the subject is that of Morrison (2) who found the iron content of fourteen samples of dialyzed, dried preparations of human hemoglobin to be from 0.305 to 0.338 per cent. Morrison and Hissey (3) showed that human hemoglobin containing 1 atom of iron will bind 1 mole of carbon monoxide.

The investigation described here was undertaken to determine one of the primary constants of crystalline human hemoglobin; namely, its iron content.

## Methods

*Crystalline Human Hemoglobin*—Hemoglobin from a composite sample of red blood cells, obtained from about twenty adult individuals, was crystallized by the method of Cannan<sup>1</sup> and recrystallized twice. After the last recrystallization the crystals were dissolved in a minimum of water and dialyzed until tests for inorganic ions were negative. The dialyzed hemoglobin solution was then evaporated and dried at 105° in an electric oven to constant weight. After being ground and reheated at 105°, the powdered hemoglobin was stored in a desiccator over phosphorus pentoxide.

*Iron Determination*—Accurate volumetric determination of the iron content of hemoglobin and other biological materials has usually been done by the titanium reduction method (2-6). This method has three disadvantages: an unstable standard is used for the titration, oxygen must be excluded during the titration and from the stored titanium solution, and finally, chlorides interfere in the determination. The interference of chlorides precludes the use of hydrochloric acid, which is the best solvent for the iron ash. This is a point of practical importance in dry ashing procedures.

An accurate and rapid method, free of these difficulties, for the estimation of 5 to 15 mg. of iron, was developed by semimicro adaptation of the

<sup>1</sup> Cannan, R. K., personal communication.

widely used procedure of titration of ferrous iron with potassium dichromate (7).

*Reagents—*

Sulfuric acid, iron-free, 36 N.

Hydrochloric acid, 12 N.

Phosphoric acid, 85 per cent.

Stannous chloride solutions. 2 gm. of iron-free  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in 100 ml. of 6 N HCl. *It is important that this solution be freshly prepared.*

Mercuric chloride solution. 5 per cent solution of  $\text{HgCl}_2$  in water.

Indicator solution. A 0.2 per cent solution of barium diphenylamine sulfonate in water.

Standard potassium dichromate solution. An approximately 0.028 N solution with potassium dichromate prepared by recrystallizing the reagent grade product and drying the pulverized crystals at 150–200° in an electric oven.

*Analytical Procedure—*About 2 to 5 gm. of hemoglobin were weighed by difference, with precautions against absorption of moisture, into a tall porcelain crucible of 50 ml. capacity. 2 ml. of sulfuric acid were added and the crucibles heated in an electric oven, starting at 80° and increasing to a maximum of 135° over a period of 8 hours.

The crucibles were then placed on a hot-plate and heated until the contents stopped bubbling. Finally the crucibles were placed in a cold muffle furnace. The temperature of the furnace was brought to 590° in about 2 hours and heating was continued for 8 hours. After 2 ml. of 12 N hydrochloric acid had been added to the dry ash, each crucible was covered with a watch-glass and placed in a steam bath for an hour. The cover-glass was removed and the solution evaporated to approximately 0.2 ml.; then 1.5 ml. of water were added and the solution heated almost to boiling on a hot-plate. The stannous chloride solution was added drop by drop from a capillary pipette, with agitation, until the yellow color of the ferric iron had disappeared; then one more drop was added. The solution was cooled in a water bath to below 25° and 1 ml. of 5 per cent mercuric chloride solution was blown in all at once. The determination was discarded if the precipitate which was obtained was not white, silky, and small in amount. At this point 15 ml. of water, previously measured out, 1.5 ml. of 7 N sulfuric acid, 0.5 ml. of 85 per cent phosphoric acid, and 0.04 ml. of indicator solution were added as rapidly as possible and the mixture titrated at once with standard potassium dichromate solution, with mechanical stirring, until a violet tinge appeared.

With the reagents we employed, the indicator and reagent blank was equivalent to 0.013 ml. of 0.01 N potassium dichromate.

*Accuracy of Method—*All weights and volumetric apparatus were cali-

brated. 10 ml. of an iron solution containing 8.935 mg. of iron, prepared from iron wire (99.8 per cent iron) and dilute hydrochloric acid, were evaporated to 0.5 ml. in a porcelain crucible and the iron content determined. The average value of eight consecutive determinations was 8.934 mg., with an average error in a single determination of 0.011 mg.

### Results

The determination of the iron content of hemoglobin and determinations designed to show whether or not a loss of iron occurred during the ashing procedure are shown in Table I.

TABLE I

*Iron Content of Hemoglobin and Recovery of Known Amounts of Iron after Ashing*

Sample No.	Material	Iron added	Iron found	Per cent iron in hemoglobin
		mg.	mg.	
1	Iron solution	8.935	8.900	
2	" "	8.935	8.945	
3	" "	8.935	8.930	
4	" "	8.935	8.945	
5	Hemoglobin, 2.5211 gm.		8.588	0.3406
6	" 2.5951 "		8.821	0.3399
7	" 2.3408 "		7.945	0.3400
8	" 2.6712 "		9.087	0.3402
9	" 2.7629 "		9.414	0.3407
10	Iron solution + 3 gm. glucose	8.935	8.930	
11	" " + 3 " "	8.935	8.941	
12	" " + 3 " "	8.935	8.941	
13	Hemoglobin, 4.1915 gm.		14.27	0.3405
14	" 4.2158 "		14.31	0.3395
15	" 4.3094 "		14.64	0.3398
16	" 4.5264 "		15.35	0.3391
17	" 4.4629 "		15.18	0.3401
Average.....				0.340
" error of single determination.....				0.0004

### DISCUSSION

The value of 0.340 per cent, obtained for the iron content of human crystalline hemoglobin dried at 105°, corresponds to a minimal molecular weight of 16,400 and an oxygen capacity of 1.36 ml. per gm. These values for the constants of hemoglobin indicate that the primary constants assumed in hemoglobin methods are 1.5 per cent too low; hence the concentrations reported as gm. of hemoglobin per 100 ml. of blood are too high by the same percentage.

We wish to express our thanks to Dr. R. L. Haden for suggesting this problem, to Dr. R. Keith Cannon for providing his method for the crystallization of human hemoglobin, and to Dr. D. Roy McCullagh for supplying red blood cells.

#### SUMMARY

1. An accurate method of determining 5 to 15 mg. of iron is presented.
2. The iron content of crystalline human hemoglobin dried at 105° was found to be 0.340 per cent.

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# SOLUBILITY OF NITROUS OXIDE IN HUMAN BLOOD\*

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The solubility of nitrous oxide in blood at body temperature was determined by Siebeck (1) with considerable accuracy as early as 1909. In his experiment, the blood was placed with a gas mixture in a tonometer of 3 liters capacity and equilibrated at 38° and atmospheric pressure. After an equilibrium had been attained, samples of both blood and gas were analyzed for nitrous oxide content. A blood cell solution was used which had been prepared from beef blood and was considered by Siebeck to be equivalent to whole blood with respect to N<sub>2</sub>O solubility.

On three 100 cc. samples of this solution equilibrated at 38° with a known gas mixture, Siebeck obtained  $\alpha$  values of 41.08, 42.73, and 41.29, respectively, averaging 41.7 or 0.417 per cc. This  $\alpha$  is the Bunsen absorption coefficient described (2) as "the volume of gas (reduced to 0° and 760 mm.) taken up per unit volume of solvent when the pressure of the gas itself minus the vapor tension of the solvent is 760 mm."

In spite of the fact that the substitution of such a blood cell solution for whole blood may be open to question, the  $\alpha$  value of 0.417 agrees very closely with that found by Orcutt and Seevers ( $\alpha = 0.416$ ), who have recently described a new method for determining the solubility of gases in liquids (2). This method, specifically applied to nitrous oxide in blood (3), may be considered the most accurate at the present time, and has been used in the experiments now to be reported to determine the solubility of nitrous oxide in blood at 37.5°.

## Methods

Oxalated venous blood samples were equilibrated in a tonometer at 37.5° and atmospheric pressure with gas mixtures of varying concentrations of N<sub>2</sub>O, N<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub>. The volumes of blood and gas used, the length of equilibration, the method of sampling, and other details of procedure were the same as those described for normal oxygen tension determinations (4).

Blood samples (1 cc.) were analyzed according to the method previously mentioned (3). Gas samples (25 to 30 cc.) were analyzed by the Van Slyke manometric procedure (5), corrections being made for the reabsorption of nitrous oxide in the sodium hydroxide and hydrosulfite reagents.

\* Aided in part by a grant from Linde Air Products, New York.

TABLE I

*Composition, Barometric Pressures, and Nitrous Oxide Tensions of Blood and Gas Samples*

Sample No.	Equilibrated gas, per cent composition				Barometric pressure	$pN_2O$	$N_2O$ in equilibrated blood
	$CO_2$	$O_2$	$N_2$	$N_2O$			
					mm. Hg	mm. Hg	vol. per cent
1	5.95	9.48	0	84.5	740	583	30.9
2	6.15	4.21	0	89.6	737	616	33.2
3	6.30	1.04	4.16	88.5	738	609	34.0
4	5.75	17.40	0	76.8	740	531	28.7
5	6.10	5.33	21.30	66.3	740	458	25.2
6	5.30	46.80	0	47.9	738	330	17.8
7	5.60	9.50	38.00	46.9	738	323	17.7
8	5.80	1.66	0	92.5	740	639	34.0
9	1.23	0.60	0	98.1	740	678	37.4
10	5.80	33.70	0	60.5	738	417	22.0
11	5.70	7.50	30.00	56.8	737	391	21.8
12	5.25	75.80	0	19.0	736	131	7.4
13	5.38	15.60	62.40	16.6	736	114	6.6
14	0.42	0.40	0	99.1	736	681	37.6
15	5.45	16.25	65.00	13.3	738	92	4.8
16	5.33	63.80	0	30.8	742	213	11.7
17	0.57	0.28	0	99.1	745	690	37.9
18	5.53	53.60	0	40.8	738	281	15.1
19	6.44	11.00	44.00	38.5	738	265	14.2
20	5.71	39.60	0	54.7	737	376	19.9
21	5.63	8.12	32.48	53.7	736	369	20.9

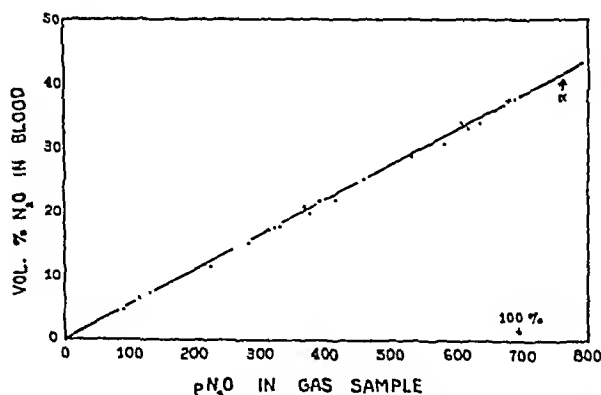


FIG. 1. Nitrous oxide content of human blood when equilibrated at 37.5° with varying tensions of nitrous oxide.  $pN_2O$  is expressed in mm. of Hg.

### Results

The analyses of blood and gas samples are recorded in Table I, together with the barometric pressures and corresponding nitrous oxide tensions which were calculated by the formula:

$$pN_2O = (\text{barometric pressure minus vapor pressure of blood}) \% N_2O \text{ in gas sample}$$

When the volume of nitrous oxide found in blood is plotted against the nitrous oxide found in gas samples, the points fall very nearly in a straight line, as is shown in Fig. 1. Since the volume of nitrous oxide in blood is calculated in terms of 0° and 760 mm., it is possible to read the  $\alpha$  value directly from the graph, the  $\alpha$  value being 0.415.

### DISCUSSION

Since the blood and gas in the tonometer were at atmospheric pressure (approximately 740 mm.) and since the vapor pressure of blood is about 49 mm., a gas sample of 100 per cent nitrous oxide would exert only about 691 mm. pressure. However, to determine the amount of nitrous oxide that would be dissolved in blood at 37.5° when  $pN_2O = 760$  mm. of Hg, it is permissible to read the value from the line in the graph. This value would be  $\alpha$  and is about 0.415, which agrees very closely with the values previously mentioned (2).

Seever and Waters (6) give the solubility of nitrous oxide in terms of Ostwald's solubility expression (2) and report  $\lambda = 0.470$  for blood at 37.5°. To convert  $\lambda$  to  $\alpha$ , one can apply the simple formula,  $\lambda = \alpha(1 + 0.00367t)$ . When the  $\alpha$  value found in the present experiment is used,  $\lambda = 0.415(1 + 0.00367 \times 37.5) = 0.472$ .

Numerous investigators (7-15) have reported nitrous oxide content of blood during anesthesia, but because of differences in methods of analysis, time of sampling, and lack of data on composition of inspired gas mixtures, the results are quite confusing. All of the results are lower than the average value of 30 volumes per cent found by the authors in the blood of patients inspiring approximately 80 volumes per cent of nitrous oxide (7). Smith (8) who used the same method as was used in the present experiments, obtained 28 volumes per cent, which agrees more closely than any of the others.

It must be remembered that in the alveoli vapor tension exerts a tension of about 49 mm. of Hg, and  $CO_2$  at least 40 mm., leaving only 650 mm. tension (barometric pressure 740 mm.) for nitrous oxide, if 100 per cent nitrous oxide were being inspired. In other words, a patient breathing 100 per cent nitrous oxide (if he lived long enough to attain equilibrium between alveoli and blood) would have from 35 to 36 volumes per cent of nitrous oxide in his blood. This agrees rather closely with Jolyet and



Blanche (15) who found 34 and 37 volumes per cent in blood of two dogs after they had breathed 100 per cent nitrous oxide for 3 minutes.

The elimination of nitrous oxide from blood while air is breathed must necessarily depend somewhat upon the rate of respiratory exchange of the patient. It is doubtful, however, that the nitrous oxide would be completely eliminated in 5 minutes as reported Nieloux (11). When an arterial blood sample was drawn from a patient 20 minutes after anesthetic gas had been discontinued, it was found by the authors to contain from 1 to 2 volumes per cent of nitrous oxide. Although this amount is negligible from an anesthetic standpoint, it is still a definitely measurable amount.

#### SUMMARY

The solubility of nitrous oxide in blood at 37.5° was found to be  $\alpha = 0.415$  and  $\lambda = 0.472$ .

A brief summary is given of the nitrous oxide content of arterial blood during nitrous oxide anesthesia.

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# CATECHOLASE (TYROSINASE):\* REVERSIBLE INACTIVATION AND REACTIVATION†

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Studies of the chemical properties of the enzyme, catecholase, have shown that this polyphenoloxidase consists of a copper-protein complex (1-3). Indications have also been obtained that the enzymic activity of catecholase is associated with the copper part of the molecule, since it has been found that the addition of certain reagents known to react with cupric ions will inhibit the enzymic activity of catecholase preparations (1, 2, 4, 5). We were interested to find out whether the enzyme, so inactivated, could be reactivated by the addition of certain metal ions. Cupric, ferric, cobaltous, and manganous ions were tested. In the following we wish to report on our findings.

## EXPERIMENTAL

*Preparation of Enzyme*—The catecholase preparation, used in the inactivation studies, was obtained from the common mushroom, *Psalliota campestris*, according to the method of Tenenbaum and Jensen (6). The preparation had an activity of about 250 Adams and Nelson units per mg. of dry organic weight, a unit being defined as that amount of enzyme which will cause an oxygen uptake of 10 c.mm. per minute when the catechol-hydroquinone substrate (7) is used.

*Inactivation Experiments*—The first series of experiments was carried out in order to determine the effect of time on the inactivation of the enzyme by the addition of the reagents, potassium cyanide, sodium diethyldithiocarbamate, and potassium ethyl xanthate, and also the degree of reactivation following the addition of cupric ions to the inactivated enzyme solution.

The following solutions were prepared.

*Solution 1*—This was a solution containing enzyme, buffer (phosphate-citrate, 0.4 M, pH 6.5), and gelatin, made up to contain 1 ml. of the buffer and 5 mg. of gelatin for every 1.3 units of enzyme in the solution.

\* The term catecholase is preferable to tyrosinase, but the latter, however, is still in general use.

† A report upon this work was presented at the meeting of the American Chemical Society at Buffalo, September, 1942, before the Division of Biological Chemistry.

*Solution 2*—This solution prepared as described for Solution 1 contained in addition 0.1 mg. of inactivating agent for every 1.3 units of enzyme.

*Solution 3*—This solution was prepared as described for Solution 2, but contained in addition 0.5 mg. of cupric ion (added as a solution of cupric chloride) for every 1.3 units of enzyme. The copper salt was added immediately after the addition of the inactivating agent.

In determination of the enzymic activity, an aliquot containing 1.3 units of enzyme was withdrawn from the desired solution for the test.

After the solutions were prepared, they were allowed to stand at room temperature for 10 minutes and then tested. They were then placed in the refrigerator and tested 24 and 72 hours afterwards. The solution containing the active enzyme (Solution 1) was run as a control to determine the changes in the enzymic activity which occur on standing. The solution containing the enzyme and inactivating agent (Solution 2) was tested to determine the loss in potency due to inactivation. An equivalent portion of Solution 2 was tested at the same time in the presence of 0.5 mg. of cupric ion (added just before the determination) to determine the amount of reactivation which could be obtained after the enzyme had been in contact with the reagent for the given length of time. Solution 3, which contained enzyme, inactivating agent, and cupric ion, was tested to determine the protective influence of the cupric ion when it was added immediately after the addition of the inactivating agent.

A second series of experiments was carried out to ascertain the effect of other metal ions on the degree of reactivation of a previously inactivated enzyme solution. The metal ions besides cupric used in these experiments were ferric, manganous, and cobaltous and were added as solutions of their corresponding chlorides.

For this test, the enzyme solutions were prepared as described for Solution 2 and allowed to stand for 10 minutes. At the end of the 10 minute period 0.5 mg. of the desired ion (cupric, ferric, manganous, or cobaltous) was added and the solution tested. The oxygen uptake given by the inactivated enzyme solution to which the metal ion had been added was compared with the uptake obtained with an inactivated enzyme solution to which no metal ion had been added. A similar series of experiments was undertaken in which a 0.4 M acetate buffer (pH 5.9) was used in place of the phosphate-citrate buffer. Controls were run to determine whether the metal ions, themselves, would cause an oxygen uptake; none was observed when the quantities mentioned above were used.

#### DISCUSSION

As can be seen from Table I, it was found in agreement with other investigators (1, 2, 4, 5) that potassium cyanide, sodium diethyldithio-

carbamate, and potassium ethyl xanthate will inhibit catecholase activity. The addition of cupric ions will restore enzymic activity, the degree of reactivation apparently depending upon the time which elapses between the addition of the inactivating agent to the enzyme solution and the addition of the cupric ion. When cupric ions were added immediately following the addition of the inactivating agent, a small loss in activity occurred, which, however, did not increase upon standing. Apparently the addition of cupric ions prevents any further inactivation from taking place (see the last two columns in Table I). It appears that longer contact

TABLE I

*Effect of Time on Reactivation of Inactivated Catecholase by Cupric Ions*

0.1 mg. of the agent and 0.5 mg. of copper were used in these experiments. The oxygen uptake is given in c.mm. for a run of a half hour duration. The per cent reactivation is calculated on the basis of the amount of activity which still remains after inactivation, and not on the total amount of activity.

Inactivating agent	Time	Catecholase alone	Catecholase + inactivating agent		Catecholase + inactivating agent + cupric ions added just before test		Catecholase + inactivating agent + cupric ions added immediately after agent had been added	
		Oxygen uptake	Oxygen uptake	Per cent inactivation	Oxygen uptake	Per cent reactivation	Oxygen uptake	Per cent reactivation
Potassium cyanide	10 min.	424	3.5	99	304	72	335	79
	24 hrs.	420	5.0	98	236	56	342	81
	72 "	408	3.8	99	178	44	328	80
Sodium diethyl-dithio-carbamate	10 min.	402	11.4	97	280	70	375	93
	24 hrs.	396	0	100	100	25	358	90
	72 "	384	0	100	45	12	350	91
Potassium ethyl xanthate	10 min.	411	55	87	379	79	402	98
	24 hrs.	406	48	88	271	55	388	96
	72 "	392	44	89	228	47	370	94

of the inactivating reagent with the enzyme in the absence of cupric ion produces some irreversible inactivation.

The presence of gelatin in the reaction mixture did not exert any qualitative influence on the degree of inactivation and reactivation; however, the oxygen uptake was less in the absence of gelatin.

As can be seen from Table II, addition of cupric ions gives considerably more reactivation than any of the other metal ions employed. Ferric and cobaltous ions cause little reactivation except in the case in which the enzyme was inactivated by sodium diethyldithiocarbamate. Manganous ions produced some reactivation when the buffer medium was phosphate-citrate, but in the acetate buffer no reactivation was observed.

Potassium ethyl xanthate and sodium diethyldithiocarbamate gave about the same amount of inactivation in the presence of either phosphate-citrate or acetate buffer; however, potassium cyanide did not produce sufficient inactivation in the presence of acetate buffer and a larger quantity (0.3 mg.

TABLE II

*Reactivation of Inactivated Catecholase by Various Metal Ions*

E. = enzyme (catecholase), A. = inactivating agent. The oxygen uptake is given in c.mm. for a run of a half hour duration. The per cent reactivation is calculated on the basis of the amount of activity which still remains after inactivation, and not on the total amount of activity. When no reactivation or further inactivation occurs upon the addition of a metal ion, the per cent reactivation is given as zero.

Inactivating agent	Solution tested	In phosphate-citrate buffer			In acetate buffer		
		Oxygen uptake	Per cent inactivation	Per cent reactivation	Oxygen uptake	Per cent inactivation	Per cent reactivation
Sodium diethyldithiocarbamate, 0.1 mg.	E.	390			373		
	" + A.	8	98		0	100	
	" + " + $\text{Cu}^{++}$	265		68	242		63
	" + " + $\text{Fe}^{+++}$	98		25	60		16
	" + " + $\text{Co}^{++}$	172		44	149		40
Potassium ethyl xanthate, 0.1 mg.	" + " + $\text{Mn}^{++}$	209		54	0		0
	"	393			349		
	" + A.	51	87		35	90	
	" + " + $\text{Cu}^{++}$	323		69	252		62
	" + " + $\text{Fe}^{+++}$	51		0	16		0
Potassium cyanide, 0.1 mg.	" + " + $\text{Co}^{++}$	45		0	103		19
	" + " + $\text{Mn}^{++}$	194		36	31		0
	"	387			370		
	" + A.	38	90		227	39	
	" + " + $\text{Cu}^{++}$	307		71	270		12
Potassium cyanide, 0.3 mg.	" + " + $\text{Fe}^{+++}$	60		6	179		0
	" + " + $\text{Co}^{++}$	69		8	268		11
	" + " + $\text{Mn}^{++}$	172		35	261		9
	"				368		
	" + A.				27	93	
	" + " + $\text{Cu}^{++}$				261		64
	" + " + $\text{Fe}^{+++}$				74		13
	" + " + $\text{Co}^{++}$				85		16
	" + " + $\text{Mn}^{++}$				33		0

in place of 0.1 mg.) of potassium cyanide had to be used in the presence of that buffer. This is probably a pH effect (the pH of acetate buffer is 5.9 and that of phosphate-citrate is 6.5), since it was observed that less inactivation was produced by potassium cyanide when a phosphate-citrate buffer of lower pH was used.

From the observations reported in this paper and those of other investigators, it appears that the tyrosinase molecule may be considered as being composed of a more or less separable metallic component, copper, which is bound presumably by coordinate bonds to a protein of specific nature. The copper part of the molecule is probably the chief "anchoring" group. The "fixing" of the free bonds of the copper part of the enzyme by the addition of certain reagents leads to inactivation. The liberation of these bonds by the addition of certain metal ions, especially cupric, leads to reactivation. It is probable that the linkages between cupric ion and these reagents are less dissociable than the linkages between these reagents and the other metal ions. It is unlikely that any loss in activity and its reversal are due to first reduction and then oxidation of the cupric part of the molecule. The irreversible inactivation which appears on standing may be caused by the action of the reagents on the protein portion of the molecule.

#### SUMMARY

Potassium cyanide, sodium diethyldithiocarbamate, and potassium ethyl xanthate were found to inhibit catecholase activity.

Addition of various metal ions indicated that cupric ions gave the greatest percentage of reactivation.

The longer the period of time which elapses between the addition of inactivating agent and addition of cupric ions, the smaller the amount of reactivation.

The significance of these findings in relation to the chemical structure of the enzyme, catecholase, has been discussed.

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# BLOOD CHANGES FOLLOWING GLUCOSE, LACTATE, AND PYRUVATE INJECTIONS IN MAN\*

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The metabolism of pyruvic acid has been extensively investigated *in vitro*, but few studies have been made *in vivo*. It has been shown that pyruvate can be converted to glucose in the rabbit (1) and to lactate in the rabbit and the dog (1-5). Furthermore, injected pyruvate disappears rapidly from the blood in the dog (5) and the rabbit (2). The injection or ingestion of glucose is followed by a rise in the blood pyruvate in the dog (5) and in man (6). In the present experiments we have attempted to estimate the interrelationship of intravenously injected glucose, lactate, and pyruvate in humans, and the effect of insulin on some of these reactions.

## EXPERIMENTAL

Male subjects for these studies were chosen from the wards of the Psychiatric Division of Bellevue Hospital; in these subjects (unless otherwise noted) the complaint consisted of some situational difficulty, and there was no evidence of physical disorder. Their ages varied between 19 and 37 years, and weight between 63 and 72 kilos. The study was started 18 hours after the last meal and after at least  $\frac{1}{2}$  hour of rest in bed. The blood was drawn into a syringe containing sufficient iodoacetate to make a 1 per cent solution, delivered into sampling bottles containing sufficient fluoride and oxalate to make a 1 per cent and a 0.2 per cent solution respectively, and precipitated immediately. All determinations were corrected for the dilution by the iodoacetate solution. Various quantities of glucose, sodium *dl*-lactate, sodium *d*-lactate, and sodium pyruvate were injected intravenously. The blood samples taken at intervals were precipitated with trichloroacetic acid for the determinations of pyruvic acid and bisulfite-binding substances, and with zinc sulfate and sodium hydroxide (7) for the determination of lactic acid and glucose. Methods for the analysis of glucose, pyruvic acid, lactic acid, and bisulfite-binding substances (B. B. S.) were the same as those described in previous publications (6, 8). The values for non-pyruvic acid B. B. S. were ob-

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tained by subtracting the mg. per cent of pyruvic acid obtained with the hydrazone method from the total B. B. S. (expressed as mg. per cent of pyruvic acid).

### Results

Following the administration of 15 gm. of glucose to eight subjects, there was a slight rise of both lactic acid and pyruvic acid in the blood. The increases were sufficiently large to be significant with the methods used. Similar experiments were then performed on twenty subjects with 100 gm. of glucose. The increase in lactate and pyruvate in these cases was at least twice the change observed in the patients given 15 gm. of glucose (Table I). The maximum rise in both lactic and pyruvic acids occurred at between 30 and 45 minutes. The sustained elevation of pyruvic acid after glucose injection should be noted, since injected pyruvic acid disappears rapidly from the blood *in vivo*. It is therefore probable that there is a continuous large production of pyruvate to maintain the elevated pyruvate levels in the blood. The rise in lactate and pyruvate after glucose injection was associated with no significant change in the pyruvate-lactate ratio. This is in agreement with the recent findings of Stotz and Bessey (9). The urine contained from 6 to 18 per cent of the injected glucose.

A similar study was made on ten subjects who received 100 gm. of glucose and insulin intravenously. The average increase in blood pyruvate was not significantly different from that observed in the twenty subjects who had received 100 gm. of glucose without insulin (Table I). This is in contrast to observations on diabetic dogs (10) and subjects (11) in which insulin produced a marked rise in blood pyruvate after a single glucose administration. It is suggested that the failure of insulin to produce a further rise in blood pyruvate in normal subjects is due to the marked fall in blood sugar which occurs, and effectively removes glucose for oxidation. This fall in blood sugar after insulin administration might be partly brought about either by increased glycogenesis in the muscle or by decreased glycogenolysis in the liver or by both.

When an intravenous injection of 50 to 100 gm. of glucose was followed by a continuous infusion of a 10 per cent glucose solution in saline (60 to 100 gm. of glucose per hour), a sustained rise in blood pyruvate occurred, reaching a constant level within 30 minutes. The intravenous injection of various doses of insulin after 1 to 1½ hours of glucose infusion produced a further rise in blood pyruvate (Fig. 1). This was probably due to the ample supplies of glucose available for oxidation.

The results after the intravenous injection of 18.8 gm. of racemic sodium lactate (12 per cent solution) are presented in Table II. It may be seen

TABLE I

*Changes in Blood Pyruvic and Lactic Acids after Intravenous Injection of 100 Gm. of Glucose*

The results are expressed in mg. per cent.

Subject No.	0 min.	15 min.	30 min.	45 min.	60 min.	90 min.
Glucose						
Average change (20 subjects)		+382	+254	+227	+129	+41
Average change after glucose + 20 units insulin (10 subjects)		+352	+141	+32	+8	-22

## Pyruvic acid

1	1.15	1.44	1.46	1.69	1.74	1.77
2	1.02		1.59		1.75	1.37
3	1.17		2.04	1.96	1.86	1.39
4	0.97	1.25	1.62		1.54	
5	0.91	1.64	1.74		1.96	1.33
6	1.19	1.37	1.56	1.86	1.69	1.70
7	0.80	0.96	1.08	1.23	1.20	1.06
8	0.93	1.45	1.86	1.66	1.45	1.30
9	0.92	1.01	1.50	1.66	1.64	1.07
10	0.78	1.04	1.31		1.37	0.90
11	1.10		1.58	2.20	1.46	1.43
12	0.74		1.62	1.52	1.33	0.93
13	0.76		0.82	1.18	1.02	0.92
14	0.68		1.23	1.29	1.15	0.99
15	0.78		1.47	1.37	1.82	1.44
16	1.14		1.39		1.70	1.15
17	1.04		1.59	1.62	1.62	1.05
18	1.00		1.31	1.26	1.26	1.09
19	1.10		1.29	1.49	1.50	1.07
20	0.90		1.63	1.51	1.57	1.32
Average change		+0.34	+0.57	+0.64	+0.64	+0.31
“ “ after glucose + 20 units insulin (10 subjects)		+0.59	+0.62	+0.54	+0.54	+0.18

## Lactic acid

1	8.7	12.0	11.8	13.6	17.5	19.4
2	5.3		10.5		11.6	8.5
3	9.3		16.7	16.4	13.1	10.4
4	8.5	10.9	14.8		15.5	
Average change		+2.9	+5.5	+6.0	+6.5	+4.9

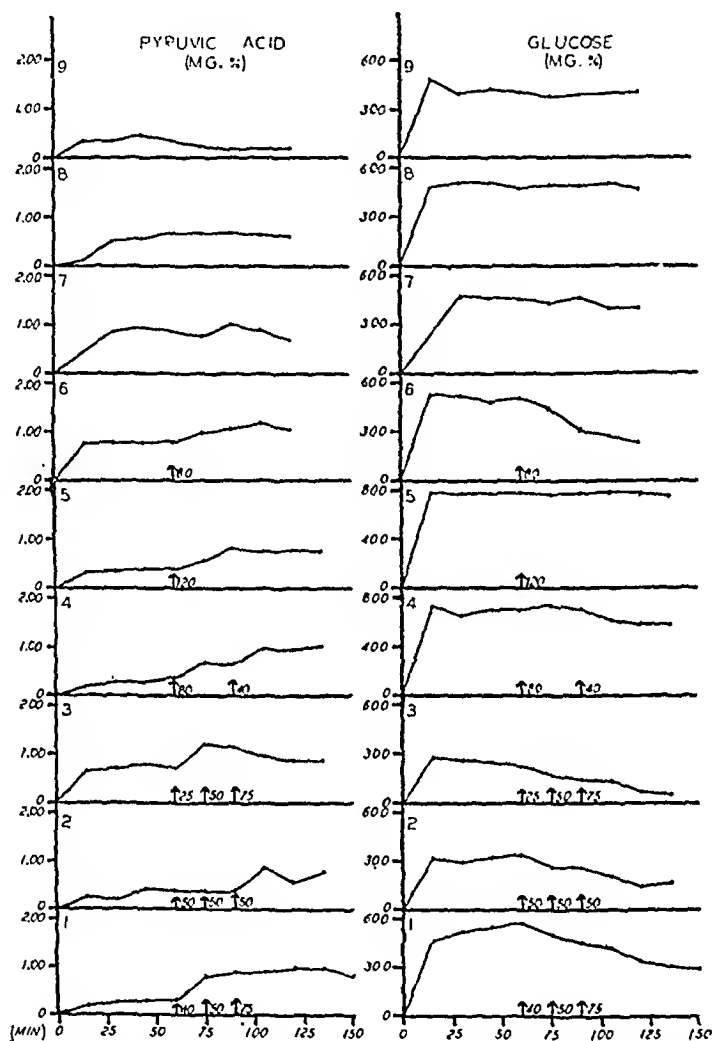


FIG. 1. Blood pyruvate and glucose curves after intravenous glucose injection immediately followed by glucose infusion. Abseissas, time in minutes after start of infusion; ordinates, increase in blood pyruvic acid and glucose (in mg. per cent) over the initial level. The arrows indicate the time at which various units of insulin were injected intravenously. Curves 1 (with insulin) and 7 (without insulin) represent observations on the same subject. The amounts of glucose given first by single injection and then during the infusion, respectively, were as follows: Curve 1, 100 gm., 100 gm. per hour; Curve 2, 100 gm., 120 gm. per hour; Curve 3, 50 gm., 60 gm. per hour; Curve 4, 60 gm., 90 gm. per hour; Curve 5, 100 gm., 120 gm. per hour; Curve 6, 50 gm., 80 gm. per hour; Curve 7, 100 gm., 100 gm. per hour; Curve 8, 75 gm., 100 gm. per hour; Curve 9, 75 gm., 100 gm. per hour.

TABLE II

*Blood Lactic and Pyruvic Acids after Injection of 18.8 Gm. of Sodium Lactate Intravenously.*

The results are expressed in mg. per cent.

Subject No.	0 min.	5 min.	10 min.	15 min.	30 min.	60 min.	120 min.
Sodium <i>dl</i> -lactate							
Lactic acid							
1	7.0			56.5	35.7	23.4	7.9
2	6.4	52.9	41.7	35.6	28.8	16.5	5.7
3	7.9		58.1	48.9	28.0	18.6	8.4
4	6.7	65.6			30.4	18.8	6.7
5	6.2	47.5	42.5	45.3	35.6	21.4	7.5
Average change		+48.9	+40.6	+39.7	+24.9	+12.9	+0.4
Pyruvic acid							
1	1.01			1.72	1.46	1.50	0.93
2	0.93	1.78	1.68	1.11	1.05	1.04	0.85
3	1.01		2.00	2.03	1.69	1.38	1.06
4	0.93	2.04	2.08	2.09	1.77	1.61	1.05
5	0.98	1.54	1.75	1.66	1.43	1.22	0.90
6	1.04	2.00	1.68	1.58			
7	0.70	1.62	1.50	1.41			
Average change		+0.88	+0.85	+0.71	+0.51	+0.38	-0.03
Sodium <i>d</i> -lactate							
Lactic acid							
1	6.5	50.0	38.8	32.1	21.5	12.0	
2	7.7	58.3	34.3	32.0	20.2	13.9	8.5
3	6.3	51.6	38.8	29.8	18.6	10.2	7.6
4	6.3	53.6		35.2	21.8	10.1	6.9
Average change		+46.7	+30.5	+25.5	+13.8	+4.9	+0.9
Pyruvic acid							
1	1.08	3.02	2.36	2.55	2.06	1.35	
2	0.85	2.59	2.48	2.44	1.73	1.47	
3	1.01	2.31	2.29	2.18	1.84	1.39	1.02
4	0.94	2.61	2.60	2.36	1.96	1.30	1.09
Average change		+1.66	+1.44	+1.41	+0.93	+0.38	+0.07

that there was a marked rise of pyruvic acid in the blood with a peak at 5 minutes after the injection. The rise in pyruvic acid exceeded that observed with both the 15 and 100 gm. doses of glucose. The excretion

in the urine of lactic acid varied from 7 to 11.5 per cent, with an average of 9.6 per cent of the injected lactate.

In view of the fact that the physiologically active form of lactate in the body is the *d* form (12, 13), we have made observations on the glucose and pyruvic acid concentrations of the blood after injection of 18.8 gm. of sodium *d*-lactate (12 per cent solution). The sodium *d*-lactate was prepared from zinc *d*-lactate (Eastman Kodak) (14). It may be seen (Table II) that the rise in blood pyruvate was about twice as great as that following the injection of the same amount of racemic lactate. The maximum rise in pyruvate occurred again at 5 minutes. Flock *et al.* (5) found a rise of pyruvate after lactate injection. This increase apparently was not considered significant. However, sodium lactate appears to be more readily converted into pyruvate than glucose, since the injection of 100 gm. of glucose did not increase the blood pyruvate as much as the 18.8 gm. of *d*-lactate did. There was no change in the blood glucose concentration after lactate injection. The rate of disappearance of the *d*-lactate from the blood exceeded that observed with racemic lactate. After the injection of 18.8 gm. of racemic sodium lactate, at least 3 times as much lactic acid is excreted in the urine (8 to 11.5 per cent of the amount injected) as after injection of the same quantity of *d*-lactate (2 to 3 per cent of the amount injected). These observations indicate again the more ready availability of the *d* form (12, 13).

Observations were made on glucose, lactate, pyruvate, and B. B. S. after injections of 18.8 gm. of sodium pyruvate (12 per cent solution) prepared according to Lu (15) immediately before injection. The subjects consisted of three non-psychotic and four schizophrenic patients. The injected pyruvate disappears much faster from the blood than the same amount of injected lactate (Table III). There was an increase in blood lactate (Table III) which reached a peak 4 minutes after the injection was terminated and was sustained for 45 minutes. The rise in lactate far exceeded that observed after the injection of glucose (Table I). It should be noted that human blood can convert pyruvate to lactate *in vitro* (16, 8). The non-pyruvic acid B. B. S. were increased during the first 45 minutes after the injection. This confirms earlier evidence (17, 2) that pyruvic acid is partially converted into other carbonyl compounds *in vivo*. This conversion does not take place in human blood *in vitro* (8). Only 3 to 4 per cent of either pyruvate (2 to 3 per cent) or lactate (1 to 1.5 per cent) was excreted in the urine. No change in blood sugar was observed. The four schizophrenic patients were treated by insulin shock and injections were made during deep coma. In order to eliminate the factor of muscular exercise, patients were chosen who went into shock quietly without struggling. It may be seen that the changes in pyruvate, B. B. S., and lactate were essentially the same. The rate of removal of injected pyruvate is

TABLE III

*Blood Changes after Intravenous Injection of 18.8 Gm. of Sodium Pyruvate*  
The results are expressed in mg. per cent.

Subject No.	0 min.	Middle of injection	4 min.	10 min.	45 min.	90 min.
Pyruvic acid						
1	1.11	16.1	9.31		2.86	1.20
2	0.96	17.8	21.5	12.4	1.80	1.28
3	1.00	27.2	19.5	16.9	2.95	1.75
4	1.14	32.6	24.7	6.70	4.52	2.17
5	1.10	22.7	13.2	8.20	1.95	1.12
6	0.92		13.0	4.82	2.02	
7	1.03	11.0		4.62	1.88	
Average change		+20.2	+15.8	+7.9	+1.53	+0.64
Lactic acid						
1	10.2	11.5	14.4		10.0	8.2
2	6.5	11.0	20.5	21.1	9.8	6.6
3	6.0	11.0	19.4	19.4	11.1	7.6
4	9.1	11.4	16.2	16.8	13.9	9.2
5	7.7	9.2	17.5	17.1	9.8	6.1
6	7.0		13.0	14.5	7.5	
7	6.3	8.2		16.3	10.4	
Average change		+2.7	+9.1	+10.4	+2.8	0.0
B. B. S. other than pyruvic acid						
1	3.08	9.50	9.3		5.06	4.34
2	4.78	7.9	6.1	6.5	4.63	4.47
3	3.68	7.2	9.6	9.8	6.79	4.17
4	4.44	8.0	13.6	10.7	6.0	7.1
5	4.38	12.8	7.6	4.9	6.1	4.6
6	3.02		8.0	6.0	5.7	3.9
7	4.04	6.7		6.3	4.7	
Average change		+4.5	+5.1	+3.3	+1.7	+0.9
Average change in insulin shock (4 subjects)						
Pyruvic acid		+21.7	+15.9	+6.7	+1.08	+0.42
Lactic "		+2.1	+8.1	+10.9	+2.9	+0.2
B. B. S. other than pyruvic acid		+5.2	+6.3	+4.6	+1.2	+0.2

therefore the same regardless of whether insulin is absent (10) or present in either normal or excessive amounts. In six additional schizophrenic patients undergoing insulin shock treatment, blood pyruvate showed, in

agreement with observations of Elliott *et al.* (18), no change during the shock despite the fact that the blood sugar fell to levels below 20 mg. per cent.

#### SUMMARY

1. In human subjects, a single injection of glucose was followed by a rise in pyruvic and lactic acids in the blood, which returned to the normal levels in about 2 hours. A continuous infusion of glucose produced a rise in blood pyruvate which was sustained as long as the infusion was continued. Insulin had no effect on the pyruvic acid curves after a single glucose injection, but produced a further rise in the pyruvic acid level during continuous glucose infusion.

2. When either sodium *dl*-lactate or sodium *d*-lactate was injected, a rise in blood pyruvic acid occurred. The rise in pyruvate was greater with sodium *d*-lactate.

3. Pyruvate injection produced a rise in blood lactate and carbonyl compounds other than pyruvic acid. Injected pyruvate disappeared more rapidly from the blood than the same amount of *d*-lactate. These changes were not affected by the injection of large amounts of insulin.

4. No changes in the glucose content of the blood after either pyruvate or lactate injections were observed.

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# THE PREPARATION OF HEXOSE DIPHOSPHATE, HEXOSE MONOPHOSPHATE, AND PHOSPHOGLYCERIC ACID\*

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In order to study the isolated component reactions of the glycolytic scheme, it is necessary to have available in pure form the phosphorylated intermediates involved in these reactions. The present paper describes the preparation of hexose diphosphate, hexose monophosphate, and phosphoglyceric acid by a method which has two advantages: (1) all three compounds are prepared from a single fermenting mixture and (2) fresh yeast is employed, thus eliminating the uncertainties of dried yeast preparations and Lebedev extracts, in addition to eliminating considerable unnecessary manipulation and testing of extracts. So far as we are aware, there have been no methods published for the preparation of the hexose phosphates from fresh yeast.

The method most commonly used for the preparation of hexose diphosphate and hexose monophosphate has been that of Robison and Morgan (1) as modified by Warburg and Christian (2). This method involves the use of Lebedev extract, which has been consistently used as the source of the phosphorylating enzymes by various workers. The fact that an inactive Lebedev extract is frequently obtained has been the cause of considerable difficulty. Since the preparation of the aqueous extracts from the same dried yeast sample on successive days often yields extracts of widely different fermenting ability, it is possible that investigators have discarded an active yeast because of their inability to obtain an active extract. The precautions which must be taken in drying the yeast in order to obtain the proper amount of autolysis of the yeast cells also suggested the desirability of elimination of these steps by using fresh yeast.

Neuberg and Kobel (3) have described a method for the preparation of 3-phosphoglyceric acid with dried yeast autolyzed for  $3\frac{1}{2}$  hours. Vercellone and Neuberg (4) found that fresh yeast treated with toluene could be substituted for dried yeast. They catalyzed the phosphorylation process by the addition of a 3 per cent solution of hexose diphosphate, used fluoride to stop the further breakdown of phosphoglyceric acid, and used acetaldehyde as the hydrogen acceptor so that phosphoglyceraldehyde was converted to phosphoglyceric acid. Ostern and Guthke (5) found that the

\* This work was aided by the Jonathan Bowman Fund for Cancer Research.



addition of hexose diphosphate was unnecessary, provided they allowed a preliminary phosphorylating period.

By analytically determining the concentrations of the three esters throughout the incubation period in a mixture similar to that used by Ostern and Guthke (5) and by varying the conditions in order to obtain a maximum yield of each of the three esters, we have been able to prepare hexose diphosphate, hexose monophosphate, and phosphoglyceric acid from the same yeast-fermenting mixture, which is prepared with fresh yeast and is therefore more easily reproduced. Experiments with both dried yeast and Lebedev extracts were carried out, and, although highly active extracts were obtained, the results were no better than with fresh yeast.

### *Method*

Bottom yeast<sup>1</sup> was washed four or five times with cold distilled water by decantation until the supernatant liquid was clear. The final supernatant liquid was decanted off, and the yeast was filtered through Filter Ccl on a Buchner funnel. The resulting pressed yeast was stored at 0°. The moisture content was about 75 per cent.

For the preparation of the phosphorus esters, 100 gm. of fresh yeast were mixed with 12.5 cc. of 10 per cent glucose (both c.p. glucose and commercial cerelese were used) and allowed to stand 15 minutes at 37°. Next, 150 cc. of 40 per cent glucose and 150 cc. of 0.67 M  $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$  buffer at pH 7.0 were added and the mixture was allowed to stand at 37° for 40 minutes. We found that pH 7.0 was optimum. Then 12.5 cc. of toluene were added and the mixture was allowed to stand at 37° for 3 hours with frequent stirring and at the end of the 3 hour period 12.5 cc. of 0.5 M fluoride were added. In measuring the concentration of hexose diphosphate at various periods of time, we noted that its concentration decreased in about 10 hours after the addition of fluoride. This decrease could be eliminated if more glucose and phosphate were added; therefore, we added one-half the original quantity of phosphate and glucose after the addition of fluoride. The mixture was then allowed to stand 12 to 20 hours at room temperature. The concentration of each of the esters was measured at various intervals during the fermentation period in order to determine the time when a maximum concentration of each of the desired esters occurred. We found, as shown in Fig. 1, that the three esters should be isolated between 12 and 24 hours after the beginning of the incubation period.

A quantity of 100 per cent trichloroacetic acid equal to 4 per cent of the total volume was then added and, after standing for 20 minutes at 0°,

<sup>1</sup> The yeast was furnished by the Fauerbach Brewing Company, Madison, Wisconsin.

the mixture was centrifuged. The toluene adhered to the precipitate, which was discarded.

Robison and Morgan (1) experienced difficulty in separating hexose diphosphate from a crude precipitate containing much inorganic phosphorus, because the solubility of barium hexose diphosphate was much decreased in the presence of inorganic phosphorus. We eliminated this possible difficulty by removing the inorganic phosphorus as magnesium ammonium phosphate. The supernatant solution was made alkaline by addition of ammonium hydroxide, and magnesium acetate was carefully added until no more precipitate was formed. The precipitate of magnesium ammonium

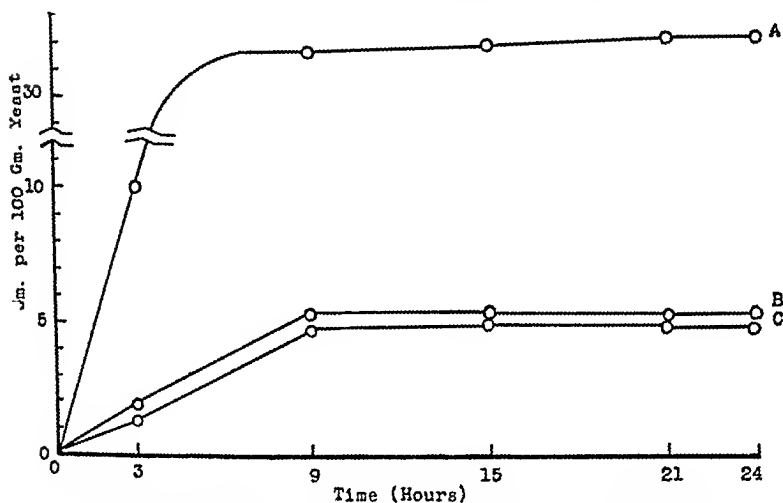


FIG. 1. Rate of formation of phosphorus esters by toluene-treated yeast. Curve A, phosphoglyceric acid; Curve B, hexose diphosphate; Curve C, hexose monophosphate.

phosphate was filtered off and discarded. Barium acetate equal to the weight of sodium phosphate used was added, and the pH was adjusted to 8.4 with  $\text{Ba}(\text{OH})_2$ . This precipitate, which we refer to as Precipitate 1, was centrifuged down, washed with alcohol and then with acetone, and dried. It consisted chiefly of barium phosphoglyceric acid and barium hexose diphosphate.

Since barium hexose diphosphate is soluble to the extent of 1 gm. in 200 cc. of solution, it is not completely precipitated by the first barium acetate treatment. If alcohol is added in a quantity equal to 10 per cent of the total volume of liquid, then the remaining barium hexose diphosphate is

precipitated (1). This precipitate was washed with alcohol, then with acetone, and dried in a vacuum desiccator. This precipitate is referred to as Precipitate 2.

Hexose monophosphate forms a soluble barium salt and therefore requires special treatment for its separation. For its isolation, the procedure of Robison and Morgan (1) as modified by Warburg and Christian (2) was used. To the supernatant from Precipitate 2, 100 cc. of solution of lead subacetate, u.s.p., were added. The lead precipitate was centrifuged down and washed with water. It was decomposed with hydrogen sulfide, and the lead sulfide was then filtered off and again treated with hydrogen sulfide. The combined filtrates were aerated thoroughly, then treated with barium hydroxide to pH 8.4, and the small precipitate which formed was filtered off and discarded. The filtrate which contained the barium hexose monophosphate was poured into twice its volume of alcohol. The precipitate was centrifuged down, washed with alcohol, then with acetone, and dried in a vacuum desiccator. This is referred to as Precipitate 3.

*Separation of Compounds in Crude Precipitates*—Since we desired to isolate the three esters from the same fermentation mixture, it was necessary to precipitate the phosphoglyceric acid and the hexose diphosphate together and then separate them by fractional solubility. Warburg and Christian (6) have described a method for separation of hexose diphosphate and phosphoglyceric acid in which the hexose diphosphate is hydrolyzed. This method was not applicable here, however, because we desired to isolate both of the compounds.

Crude Precipitate 1 was freed from hexose monophosphate by triturating it with 5 times its weight of water. The remaining precipitate was dried and then triturated with 200 times its weight of water at pH 8.4 (1). The barium hexose diphosphate was thus dissolved. To the solution of barium hexose diphosphate was added a quantity of alcohol equal to 10 per cent of the total volume (1). The barium hexose diphosphate thus precipitated was centrifuged down, washed with alcohol and then with acetone, dried, and later combined with the hexose diphosphate from crude Precipitate 2.

The remainder of the crude Precipitate 1 contained the barium phosphoglyceric acid. This precipitate was dissolved in dilute hydrochloric acid, and dilute sulfuric acid was then added to remove all of the barium. Ammonium hydroxide was then added until the mixture was alkaline, and magnesium acetate was added until no further precipitate formed. Any inorganic phosphorus which might be present was thus removed (3-5). After the filtrate was neutralized with glacial acetic acid, 15 cc. of glacial acetic acid and 12 gm. of barium acetate were added. The small precipitate which formed immediately was filtered off and discarded. The filtrate was treated with an equal volume of alcohol and allowed to stand

for 24 hours at 0°. The precipitate was filtered off, washed with water, and treated with 2 N HCl to pH 3 to 5. The insoluble portion was filtered off, washed with alcohol, then with acetone, and dried in a vacuum desiccator, yielding 3 gm. of phosphoglyceric acid with a purity of 98 per cent based on total phosphorus analyses. After 100 minutes hydrolysis, no inorganic phosphate appeared, indicating absence of hexose diphosphate.

Crude Precipitate 2 was triturated with 5 times its weight of water to dissolve the hexose monophosphate. The remaining precipitate, after drying, was triturated with 200 times its weight of water, and a quantity of alcohol equal to 10 per cent of the total volume of liquid was added. The solution was heated to 70° and filtered hot. The precipitate of barium hexose diphosphate was washed with alcohol, then with acetone, and dried. After the material was combined with the hexose diphosphate from Precipitate 1 and reprecipitated, the purity was 92 per cent, as estimated by determining spectrophotometrically the quantity of fructose present according to Roe's modification (7) of the Seliwanoff test. When organic phosphorus was calculated as hexose diphosphate, the purity was 95 per cent.

Crude Precipitate 3 was triturated with 5 times its weight of water, and the solution was added to twice its volume of alcohol. The barium hexose monophosphate was centrifuged down, washed with alcohol, then with acetone, and dried rapidly in a vacuum desiccator. The calcium salt was prepared according to the procedure of Warburg and Christian (2). The yield was 2 gm. of the barium salt which was 85 per cent pure. The purity increased to 97 per cent after reprecipitation from alcohol.

The sodium salts may be prepared by adding the calculated quantity of sodium sulfate to an acid solution of the barium salts.

#### SUMMARY

1. Existing methods for the preparation of hexose diphosphate, hexose monophosphate, and phosphoglyceric acid have been combined with the necessary modifications for separation of all three of the compounds from one yeast fermentation mixture.

2. Toluene-treated fresh brewers' yeast, instead of the usual Lebedev extract, was used as the source of the fermenting enzymes.

*Addendum*—Neuberg and Lustig (8) have just published a simplified method for the preparation of calcium hexose diphosphate by which the uncertainties of dried yeast or Lebedev extract are also eliminated and in which fresh bakers' yeast is used.

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## PENICILLIN B, AN ANTIBACTERIAL SUBSTANCE FROM *PENICILLIUM NOTATUM*\*

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The interesting discovery that molds elaborate substances that are capable of inhibiting the growth of certain bacteria has recently stimulated extensive investigation of these phenomena.

In 1929 Fleming (4) reported the presence of an antibacterial substance which he called penicillin in culture media of a strain of *Penicillium notatum*. This material could be extracted from a concentrate with absolute alcohol but not with ether or chloroform. Clutterbuck, Lovell, and Raistrick (5) found that penicillin was completely extracted with ether at pH 2 but only partly at pH 7.2. These workers attributed Fleming's negative results with ether extraction to the fact that his filtrates were alkaline in reaction.

Recently, Abraham *et al.* (1, 2) obtained a purified antibacterial substance from culture media of this mold which was an acid containing nitrogen soluble in ether, acetone, amyl acetate, and chloroform. Meyer *et al.* (6) have prepared a potent chloroform-soluble acid which also contains nitrogen. It has been assumed that these investigators have been dealing with the same material characterized by its solubility in organic solvents.

In this communication we wish to report an antibacterial substance, penicillin B, produced by *Penicillium notatum*, which is insoluble in lipid solvents but readily separated from the culture medium by adsorption on benzoic acid. Upon purification the substance appears to have the properties of a protein and is highly effective *in vitro* against both Gram-negative and Gram-positive organisms. The purest preparation obtained to date is a light yellow powder soluble in water but not in fat solvents. It is stable when dry and moderately stable in solution in the pH range 2.5 to 8.

\* For convenience we shall refer to our product as penicillin B to differentiate it from the product obtained by Abraham *et al.* (1, 2) which we shall call penicillin A. Although penicillin B may be similar to "penatin" reported by Kocholaty (3), the absence of a discussion of its chemical properties prevents a comparison of the two products.

† Lalor Foundation Fellow, 1942-43.

### *Production of Penicillin B*

*Organism Employed*—Three different strains of *Penicillium notatum* which had their origin in the strain isolated by Fleming in 1929 were studied in this work. The capacities of these strains to produce antibacterial substances were compared and found to differ only very slightly. The majority of the investigations herein reported have been carried out on the culture fluids resulting from the growth of a single strain chosen from the above three.

*Medium*—The medium which we employ is that suggested by Clutterbuck, Lovell, and Raistrick (5). This is a modification of the Czapek-Dox medium and has the following composition:  $\text{NaNO}_3$  3.0,  $\text{KH}_2\text{PO}_4$  1.0,  $\text{KCl}$  0.5,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01, and glucose 40.0 gm., and  $\text{H}_2\text{O}$  in a quantity sufficient to make 1000 ml. From 1500 to 2000 ml. of this solution are placed in Erlenmeyer flasks of 2 or 3 liters capacity. These are plugged with non-absorbent cotton and sterilized in the autoclave at 15 pounds of steam pressure for 1 hour. After sterilization the pH of this medium is 4.5.

Pyrex baking dishes having internal dimensions of  $12 \times 7.5 \times 2$  inches serve as culture vessels. These trays are covered with plain glass lids which extend beyond the edges of the vessels. The trays (with lids in place) are sterilized in the hot air oven at  $165^\circ$  for  $1\frac{1}{2}$  hours. Upon cooling, they are removed to the incubation room and charged with the sterile medium, each vessel receiving a quantity of approximately 800 ml.

The inoculum is prepared from young sporulating cultures of *Penicillium notatum* on slants of Sabouraud's maltose agar by flooding the surface growth with sterile Clutterbuck's medium and emulsifying the spores together with some of the vegetative portions of the plant by means of a sterile platinum loop. This suspension is removed with a pipette and added to the sterile medium in the culture dishes in appropriate amounts. Incubation is carried out in a specially constructed room at a temperature of  $24^\circ$ .

The development of the mold culture is followed from day to day by observing the character of the surface growth, and by determining the pH and antibacterial potency of the underlying medium. All pH determinations are carried out electrometrically and the potency tests are performed as described in a later section of this report. Usually a thin surface felt of mold mycelium forms during the first 2 or 3 days of incubation and the reaction of the culture fluid becomes more acidic. The mycelium continues to develop rapidly until the 4th or 5th day, at which time it is thick, tough, and usually shows abundant sporulation. At this time the hydrogen ion concentration attains a maximum of approximately pH 2 and slight antibacterial activity is demonstrable. Further alterations in the mycelium

which consist of the wrinkling of the felt and the deepening of its green color are detectable. After about the 5th day of incubation the hydrogen ion concentration decreases slowly and the antibacterial potency increases until, at approximately the 12th day, the reaction reaches pH 4.0 and the fluid attains its maximal antibacterial potency, normally between 1250 and 5000 units per ml. Continued incubation results in a decline in antibacterial potency and a steady decrease in the hydrogen ion concentration until neutrality is reached.

The mold culture fluid is harvested when the assays indicate that it has attained a high degree of potency. Harvesting is accomplished by sliding the lids of the trays back slightly and pouring the liquid from beneath the mold felt into a carboy which is immediately removed to the refrigerator for processing.

*Method of Assay*—The sample prepared and maintained at a temperature not exceeding 15° is diluted in accordance with its anticipated potency. The diluent used is the routine sterile Clutterbuck medium which has been cooled to 15° in the refrigerator. Serial dilutions in the same type of medium are then prepared in a series of test-tubes by mixing 1 ml. of the initial dilution with an equal amount of the cold sterile medium in the first tube, transferring 1 ml. of the mixture to 1 ml. of medium in the second tube, etc. The number of such dilutions prepared is in accordance with the anticipated activity of the sample. These tubes are immediately placed in the refrigerator, where they remain until the inoculum is added.

The test organism is *Staphylococcus aureus* (F strain). A stock culture of this organism is maintained by transfer at 14 day intervals on meat extract-peptone agar slants. In the preparation of the inoculum, a small amount of such a culture is placed in 5 ml. of sterile meat extract-peptone broth and incubated for 4 hours at 37°. This culture is then appropriately diluted with the following medium (cooled to 15°) so as to result in the presence of from 2 to 3 million viable cells per ml., as determined by plate count set up at the time of dilution and read the following day: beef extract (Difco) 0.3, peptone (Difco) 1.0, lactose 1.0, and brom-cresol purple 0.0015 per cent.

1 ml. of this suspension is placed immediately in each of the tubes containing the previously diluted test samples. The final reaction is pH 6.8. These are then incubated at 37° for 15 hours. Following incubation, the tubes are examined for the presence of visible turbidity and accompanying acid production as indicated by the color of the brom-cresol purple. Such readings are clear cut and distinct.

The results are reported in terms of the number of arbitrary units of antibacterial material contained in 1 ml. of the original test sample. A



unit is considered to be that amount of material which will prevent the development of visible turbidity and the production of acid by *Staphylococcus aureus* under the above conditions. As a control, a standard sample of penicillin B is tested daily along with the unknown preparations.

### *Purification and Properties*

*Method of Preparation*—Since some batches were found to lose their activity rather rapidly, the processing of the culture medium is started as soon as possible after the harvesting. For the same reason, the entire procedure is carried out in the cold room. The harvested medium is filtered and the pH adjusted to 3.5 with 10 per cent phosphoric acid. Alcohol saturated with benzoic acid (50 ml. per liter of medium) is added slowly with adequate stirring. After the precipitated benzoic acid has settled, the supernatant liquid is siphoned off and the solids collected on a Buchner funnel. The filter cake is washed with a volume of cold water (saturated with benzoic acid) approximately equal to the volume of the cake. The filter cake is pressed as dry as possible but not dehydrated completely, since this causes inactivation. Once the material is adsorbed, it is apparently quite stable for at least several days if it is kept moist and cold.

The benzoic acid cake is then dissolved in a volume of cold acetone equal to twice the volume of the alcoholic benzoic acid used. The insoluble material is allowed to settle out and is collected by centrifugation after the clear supernatant solution is siphoned off. This crude precipitate, after being washed twice with acetone, may be dried in a vacuum desiccator or may be used directly to obtain more highly purified material.

This crude preparation may be purified in either of two ways. First it may be extracted with 3 to 4 volumes of cold water. A second extraction usually gives only about a 10 per cent increase in the yield. To this aqueous extract is added  $\frac{1}{2}$  of a volume of cold acetone. The precipitate which forms is usually relatively inactive and is discarded. A second addition of the same volume of acetone precipitates the bulk of the activity. It is centrifuged, washed with acetone, and dried in a vacuum desiccator. This is the purified penicillin B.

The alternative method is to extract the crude material first with 50 per cent acetone. This generally removes little if any of the activity. Two extractions are then made with 2 or 3 volumes of 30 per cent acetone, which usually results in the complete extraction of the active material. The acetone concentration is increased to 50 per cent and the resulting precipitate is centrifuged, washed with acetone, and dried *in vacuo* over  $\text{CaCl}_2$ . This purified product is comparable in every way to that obtained by the first method.

Thirty-five separate batches of harvested medium varying in volume

from 15 to 115 liters have been processed by this procedure. The benzoic acid removes practically all of the activity from the medium; at least, little remains after the adsorption. However, the amount of activity extracted from the crude material obtained from the benzoic acid has varied from 10 to 100 per cent with an average recovery of 50 per cent. The purification of this crude product results in the elimination of about 90 per cent of the solids and in the recovery of 50 to 100 per cent of the activity. The potency of purified penicillin B has ranged from 12,500 to 200,000 units per mg. with an average of about 50,000.

*Chemical Properties*—In view of the extraordinary potency of our product in comparison with other antibacterial agents, it seemed desirable to make a preliminary survey of the chemical properties which may be of value in the continuation of this work. This was done with the realization that the presence of impurities may be responsible for some of the reactions and that further purification may require revision of some of these findings.

The dry purified preparations retained their activity for at least 5 to 6 months. In solution between pH 3.0 and 6.8 at 25° this material is stable for at least a month but at pH 2 or above pH 8 at this temperature, or at pH 3.8 at 47°, inactivation proceeds rapidly.

Treatment with  $H_2O_2$  or  $Na_2SO_3$  at pH 7 or with HCN at pH 3.8 does not decrease the activity. However, glacial acetic acid, formic acid, concentrated phthalate buffers, and alcohol in the presence of a sufficiently high concentration of salts, cause complete loss of potency.

These purified products contain material precipitable by trichloroacetic, tannic, tungstic, flavianic, or rufanic acid. In these instances no activity remains in the supernatant solutions. It is possible to regenerate a part of the activity from the tannic acid precipitate. At pH 4.6, Hg salts produce a precipitate but the proportion of the activity removed was not investigated. Ba, Cd, Cu, Mg, and Zn salts under the same conditions do not cause precipitation. The active principle is completely salted-out from aqueous solution by two-thirds saturation with  $(NH_4)_2SO_4$  or by full saturation with  $MgSO_4$  and is not dialyzable through Visking tubing.

Penicillin B is readily soluble in water but completely insoluble in ether, chloroform, butanol, amyl acetate, methylene chloride, acetone, ethyl or methyl alcohol, pyridine, glycerol, dioxane, propylene glycol, and formamide. It is precipitated from aqueous solution by 50 per cent acetone or 66 per cent methyl alcohol.

The biuret, ninhydrin, xanthoproteic, Hopkins-Cole, Millon, Sakaguchi, Ehrlich's diazo, and Molisch tests are positive. The test for reduced sulfur with alkaline lead acetate is positive. A decrease in activity is not produced by the action of pepsin at pH 3.8, trypsin at pH 5.7, or taka-diastase or

emulsin at pH 6.8. Papain (activated with HCN) at pH 3.8 causes almost complete inactivation in 24 hours.

The isoelectric pH, determined in the apparatus described by Cohn, Irving, and du Vigneaud (7), is 4.4 and the nitrogen content of two samples was 9.7 and 8.2 per cent (Dumas). Evidence has been obtained which indicates that free amino groups are not necessary for the activity. 1 mg. of purified penicillin B per ml. of a mixture of equal parts of 2 M  $\text{NaNO}_2$  and 0.5 M acetate buffer (pH 4.5) showed no loss in activity in 30 minutes at 25° or in 2 hours at 10°. In a similar concentration in 0.2 M phosphate buffer (pH 7.2), it was not inactivated by phenyl isocyanate in 30 minutes at 25°. Likewise, formaldehyde failed to decrease the potency.

When a suspension of 10 mg. of this material in 15 ml. of anhydrous methanol is saturated with dry hydrogen chloride at 0°, a clear light yellow solution results from which the derivative can be precipitated by absolute ethyl ether. The product has no antibacterial activity. Ketene acetylation in molar acetate buffer (pH 6.4 to 7.4) causes the formation of a water-insoluble precipitate within 5 minutes accompanied by an 80 per cent loss of activity in the supernatant solution. Treatment with anthraquinone- $\beta$ -sulfonic acid or benzyl thiuronium chloride does not yield a precipitate or produce inactivation. When coupled with diazotized sulfanilic acid (as in the determination of histidine) a red-orange color is produced and the activity is destroyed.

The activity is adsorbed readily from aqueous solution by alumina gel or by passage through a column of Decalso.<sup>1</sup> In both cases elution is accomplished with 1 to 10 per cent  $(\text{NH}_4)_2\text{SO}_4$ . 1 per cent  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$  also removes the adsorbed material from the Decalso. Norit, Permutit, and benzoic acid were found unsatisfactory for use in adsorption columns.

Studies of the ultraviolet absorption spectra of three samples of penicillin B have given the results shown in Table I. The maximum absorption at 2770 Å. and the minimum absorption at 2510 Å. resemble the values found by Darby (8) for papain. Based on the results of Holiday (9), who found a double peak at 2730 and 2800 Å. for tyrosine in acid solution and a single peak for tryptophane at 2750 Å., Darby suggests that the position of the maximum absorption is due to the presence of these two amino acids in papain. According to the Millon and Hopkins-Cole tests, tyrosine and tryptophane are present in purified penicillin B.

The discrepancy between the activities and the values for  $E_{1\text{cm}}^{1\%}$  may be due to the difficulty of obtaining accurate assays or to the likelihood that inactivated material may show absorption at the same wave-lengths.

*Influence of Yeast, Brown Sugar, and pH on Culture*—Since the active material produced by our procedure was found to be insoluble in fat

<sup>1</sup> Obtained from The Permutit Company, New York.

solvents, an attempt was made to duplicate in this regard the findings of Chain and Meyer and to compare the yield of fat-soluble material with that obtained by benzoic acid adsorption. The data are shown in Table II.

TABLE I  
*Ultraviolet Absorption of Penicillin B*

Sample No.	Activity	$E_{1\%}^{1\text{cm.}}$	
		2770 Å. (maximum)	2510 Å. (minimum)
I	units per mg.		
	100,000	1.62	1.05
II	16,000-32,000	1.47	0.79
III	12,000-25,000	1.47	1.10

TABLE II  
*Influence of Yeast, Brown Sugar, and pH on Microbiol Antagonists of  
Pencillium notatum*

Harvest		Units per liter extracted		Harvest		Units per liter extracted	
Kind	Titer	Ether	Benzoic acid	Kind	Titer	Ether	Benzoic acid
	units per l.				units per l.		
Yeast.....	160,000	80,000	64,000	Brown sugar ..	160,000	12,000*	19,000
" .....	160,000	64,000	48,000	" " ..	40,000	1,000*	>350,000
" .....	<20,000	128,000	30,000	" " ..	40,000	8,000	>300,000
" .....	80,000	32,000	8,000	" " ..	<20,000	32,000	>830,000
" .....	<20,000	32,000	100,000	Routine harvested, pH 7....	80,000	64,000	64,000
" .....	<20,000	64,000		" " ..	320,000	16,000	>650,000
" .....	<20,000	<4,000	6,000				
" .....	<20,000		80,000				
" .....	<20,000	4,000	700				
Brewers' yeast†	160,000	4,000					
Bakers' " †	40,000	64,000					

\* Extracted with CHCl<sub>3</sub> according to the procedure described by Meyer *et al.* (6).

† 10 per cent fresh bakers' yeast or 5 per cent dried brewers' yeast in place of 1.5 per cent yeast extract (Difco).

The media referred to as "yeast" harvests were prepared and cultured as described by the British investigators (1) with the exception that 1.5 per cent of commercial yeast extract (Difco) was used. Those referred to as "brown sugar" harvests differed from the media of Hobby, Meyer, and

Chaffee (10) in that C and H brand brown sugar was used in place of Jack Frost brand. In these media a maximum antibacterial activity of 160 to 320 units per ml. was attained on about the 7th day of incubation at which time the pH was about 6.0.

The "yeast" media usually contained an ether-soluble antibacterial agent. The quantity of this material, which was obtained by extracting the cold medium (0°) at pH 2, was independent of the titer of the medium. The amount of activity obtained by the benzoic acid method was comparable to that obtained by ether extraction, but this material was insoluble in ether. These results seem to indicate that two separate antimicrobial substances may be produced by *Penicillium notatum* in the same culture medium. It is interesting to note that the yields by either method bore no relationship to the titer of the untreated harvest. In some instances much more activity was obtained than was indicated by the assay of the crude medium. The significance of this is not understood but it is possible that the acidification results in the liberation of the active principle or principles, since the yield by the benzoic process was greatly increased by adjusting the pH of the harvested media to 3.5. An acidity greater than this resulted in an inactivation of this material.

The results with the "brown sugar" harvests were essentially the same except that the yield by our adsorption process was generally much greater than that obtained by ether or chloroform extraction. Apparently, in this medium the benzoic acid-adsorbable principle was produced more abundantly than the fat-soluble one. Here again there was no relationship between yield and titer of the harvest.

In two instances in which our routine medium was allowed to incubate until pH 6 to 7 was reached, the ether-soluble principle could be demonstrated. This was not the case when it was harvested at pH between 4 and 5.

**Toxicity**—We have thus far been unable to prepare a potent product which is non-toxic to mice. About 0.25 mg. (representing 3000 to 50,000 units) of most preparations given subcutaneously in a single injection to a 25 to 30 gm. mouse causes death in 3 to 24 hours. However, larger quantities are tolerated when small, repeated injections are made over a period of several days. Apparently the substance does not have a cumulative toxicity.

Thus far, attempts to detoxify this substance have been unsuccessful. Treatment with nitrous acid, pepsin, norit, benzyl thiuronium chloride, and ketene did not reduce the toxicity. Fractional precipitation with acetone and with  $(\text{NH}_4)_2\text{SO}_4$ , adsorption and fractional elution from Decalso, and electrophoresis also failed to yield any product that was appreciably less toxic. While we have obtained inactive fractions which were toxic, we have not obtained an active preparation which was not toxic.

### *Bacteriological Properties*

The cultural studies which have been undertaken have dealt for the most part with purified penicillin B. The test organism in many of these investigations has been the same strain of *Staphylococcus aureus* which is employed routinely in the assay work. Other species of bacteria also have been included and several observations of bacteriological interest have been made.

It soon became evident that glucose is a necessary ingredient of the culture medium if the striking antimicrobial action of the active principle is to be observed. While very small amounts of this material inhibit the growth of *Staphylococcus aureus* in a peptone broth containing glucose, much larger quantities fail to prevent growth in a peptone medium without glucose. A somewhat less complex medium was devised for use in cultural tests. This contains no peptone and is composed of Casamino Acids (Difco) together with small amounts of nicotinic acid and thiamine. It supports the growth of *Staphylococcus aureus* but without the addition of glucose is unsatisfactory for the demonstration of the antibacterial effects of the active agent.

Experiments were carried out to determine whether other carbohydrates and related compounds might exhibit an effect similar to that of glucose. A medium containing peptone and NaCl was prepared and to it was added the carbohydrate (1 per cent) under investigation together with varying amounts of penicillin B. *d*-Glucose, *d*-galactose, *d*-xylose, and *l*-xylose enhance the antibacterial activity of penicillin B, while *d*-mannose, *d*-levulose, dulcitol, *d*-mannitol, rhamnose, inositol, sorbitol, glycerol, lactose, sucrose, maltose, and raffinose do not have enhancing action.

The four compounds which permit the demonstration of enhanced activity are not equally effective in this respect. Table III indicates the relative effectiveness of these carbohydrates as determined in the above test.

In addition to *Staphylococcus aureus*, two other organisms (*Escherichia coli* and *Salmonella schottmuelleri*) have been studied in this manner in connection with glucose, lactose, and *d*-xylose and the results obtained have been similar to those observed when *Staphylococcus aureus* was employed as the test organism.

The activity of penicillin B has been studied in relation to several different types of bacteria. The liquid medium employed in these tests (except in the case of the pneumococcus) contained 1 per cent peptone, 1 per cent glucose, and 0.5 per cent NaCl and was neutral in reaction. Table IV shows the relative degrees of activity observed with different test organisms.

From these results it is obvious that the substance is highly active against both Gram-positive and Gram-negative bacteria. These findings are in

contrast to those of Abraham *et al.* (2) and of Hobby, Meyer, and Chaffee (10), who reported that penicillin (A) was relatively ineffective against the

TABLE III

*Relative Effectiveness of Certain Carbohydrates on Antibacterial Activity of Penicillin B*

+ indicates growth of the test organism; - indicates complete inhibition of the test organism.

Carbohydrate	Dilutions of active material in peptone medium (1 part active material dissolved in following million parts of medium)							
	0.8	1.6	3.2	6.4	12.8	25.6	51.2	0
Glucose.....	-	-	-	-	-	-	+	+
Galactose.....	-	-	-	+	+	+	+	+
d-Xylose.....	-	-	-	+	+	+	+	+
l-Xylose.....	-	+	+	+	+	+	+	+
Carbohydrate-free control ..	+	+	+	+	+	+	+	+

TABLE IV

*In Vitro Activity of Penicillin B*

- indicates absence of visible growth; + indicates presence of visible growth.

Organism	Gram reaction	Concentration of antibacterial substance (1 part purified material dissolved in following million parts of medium)							
		0.8	1.6	3.2	6.4	12.8	25.6	51.2	0
<i>Escherichia coli</i> , Strain 1.....	-	-	-	-	+	+	+	+	+
" " " 3 .....	-	-	+	+	+	+	+	+	+
<i>Aerobacter aerogenes</i> , Strain 2.....	-	-	-	-	+	+	+	+	+
" " " 5.....	-	-	-	+	+	+	+	+	+
<i>Proteus vulgaris</i> , Strain 10.....	-	-	-	-	-	-	+	+	+
<i>Salmonella enteritidis</i> , Strain 52.....	-	-	-	-	-	+	+	+	+
" <i>paratyphi</i> Mears.....	-	-	-	-	-	-	+	+	+
<i>Eberthella typhi</i> Dorsett.....	-	-	-	-	-	-	+	+	+
<i>Vibrio comma</i> .....	-	-	-	-	-	-	+	+	+
<i>Brucella abortus</i> .....	-	-	-	-	-	+	+	+	+
" <i>melitensis</i> .....	-	-	-	-	-	-	+	+	+
" <i>suis</i> .....	-	-	-	-	-	-	+	+	+
<i>Salmonella schottmuelleri</i> , Strain 2.....	-	-	-	-	+	+	+	+	+
<i>Bacillus subtilis</i> .....	+	-	-	-	-	-	+	+	+
<i>Staphylococcus aureus</i> F.....	+	-	-	-	-	-	-	-	+
<i>Diplococcus pneumoniae</i> * III.....	+	-	+	+	+	+	+	+	+

\* This organism was tested in a tryptose (Difco) broth containing 1 per cent glucose.

majority of the Gram-negative bacteria tested. On the other hand, the results obtained in the present investigation with members of the *Brucella*

group and *Escherichia coli* are perhaps similar in nature to that reported by Kocholaty (3) for these organisms.

Certain studies with *Salmonella enteritidis* brought to light a fact of some interest in connection with the rôle of glucose in the present cultural studies. A simple and completely synthetic medium was prepared according to the formula of Simmons (11) except that no agar or indicator was included. This liquid medium supports the growth of the organism satisfactorily and has the following composition: magnesium sulfate 0.2, ammonium dihydrogen phosphate 1.0, dipotassium phosphate 1.0, sodium citrate 2.0, sodium chloride 5.0 gm., and distilled H<sub>2</sub>O up to 1.0 liter.

Penicillin B is ineffective in preventing the growth of *Salmonella enteritidis* in this simple medium. However, when 1 per cent glucose is added, the active substance completely prevents growth in a dilution of 1:32,000,000. This indicates that the peptone and other complex nitrogenous components of the previously described culture media are not necessarily directly antagonistic to the active material.

While the mode of action of penicillin B is not understood, it has been observed that such activity is bactericidal in nature rather than merely bacteriostatic. Subcultures from tubes of media where visible growth had been prevented by the penicillin B have yielded negative results regardless of the concentration of active substance present in the original test.

#### SUMMARY

A new antibacterial agent has been obtained from cultures of *Penicillium notatum*.

This substance appears to be protein in nature and differs markedly from the penicillin described by other investigators.

The method of preparation, assay, and the properties of this substance are described.

Although it is quite toxic to mice, it does not have a cumulative action, thereby permitting the repeated administration of small doses over a prolonged period.

Extremely small amounts of this substance are bactericidal for both Gram-positive and Gram-negative organisms. This activity is dependent upon the presence of certain carbohydrates.

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# THE EFFECT OF DIETARY CALCIUM, PHOSPHORUS, AND VITAMIN D ON THE UTILIZATION OF IRON

## I. THE EFFECT OF PHYTIC ACID ON THE AVAILABILITY OF IRON\*

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McCance and Widdowson (1) have shown ferric phytate to be very insoluble at an acidity corresponding to that which prevails in the stomach and in the small intestine. In fact, the determination of phytic acid is based on its precipitation with ferric chloride in dilute hydrochloric acid solution (1, 2). As ferric oxide, which is difficultly soluble in dilute hydrochloric acid, has been shown to be poorly absorbed (3-5), it seemed probable that ferric phytate might also be a poor source of iron for the animal. This possibility assumed more than academic interest, inasmuch as an attempt is now being made to correct the iron deficiency of patent wheat flour by the addition of ferric phytate. Furthermore, cereal grains as well as flour contain a considerable proportion of their phosphorus in the form of phytic acid (1, 6), which might affect the availability of the iron present.

### EXPERIMENTAL

In view of the above circumstances, the effect of phytic acid on the assimilation of iron was determined in two series of experiments with rats. In the first series, phytic acid was added to a "synthetic" iron-low ration which had been supplemented with ferric chloride. In the second series, ferric phytate was prepared as such and then fed in a basal diet of whole milk.

The "synthetic" iron-low ration was a modification of the diet compounded by Schneider and Steenbock (7). It was composed of heated egg white<sup>2</sup> 18, cottonseed oil<sup>3</sup> 5, and vitab<sup>4</sup> 4 parts with additions of variable amounts of a modified Wesson's salt mixture and cecelose to make 100. The basal salt mixture was composed of NaCl 105, KCl 120, MgSO<sub>4</sub>·-

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison.

<sup>1</sup> This term is used with full realization of its limitations.

<sup>2</sup> Obtained from the Steiu-Hall Company, Chicago.

<sup>3</sup> Wesson oil.

<sup>4</sup> A rice bran concentrate obtained from the National Oil Products Company, Harrison, New Jersey.

7H<sub>2</sub>O 90, MnSO<sub>4</sub> 0.20, K<sub>2</sub>Al<sub>2</sub>(SO<sub>4</sub>)<sub>4</sub>·24H<sub>2</sub>O 0.09, NaF 0.57, KI 0.05, and CaCO<sub>3</sub> 219.44 parts. 2.14 per cent of this mixture was fed when phytic acid was the source of phosphorus. When phosphates were the source of phosphorus, the basal salt mixture was supplemented with an equimolecular mixture of 137.84 parts of KH<sub>2</sub>PO<sub>4</sub> and 176.44 parts of K<sub>2</sub>HPO<sub>4</sub>. The amount of salts incorporated in the diet was then increased to 3.40 per cent.

In realization of the fact that a dietary excess of calcium has a depressing effect on the utilization of iron, the amount of this element fed was adjusted to an optimal level. The level of calcium selected was based on the data of Krieger and Steenbock (8). While these authors obtained good growth with 0.257 per cent of calcium in the presence of optimal phosphorus, calcification of the bones was not entirely normal. In view of this, the amount of calcium was increased to 0.35 per cent for an optimal intake. In some experiments it was fed at 4 times the optimal level. This increased the percentage of salts in the ration to 4.77 and 6.03 when the diet contained phytic acid and potassium phosphates, respectively. Similarly, the amount of phosphorus added, namely 0.25 per cent of the ration, was based on the data of Krieger, Bunkfeldt, and Steenbock (9).

The calcium carbonate available from commercial sources<sup>5</sup> was found to contain too much iron for use in this experiment; *viz.*, 167  $\gamma$  per gm. This was reduced to 7  $\gamma$  per gm. by the following treatment: The calcium carbonate was dissolved in a minimal amount of dilute hydrochloric acid. After the addition of a small amount of calcium carbonate until no further solution occurred, hydrogen sulfide was passed in for complete precipitation of the iron. After filtration and removal of the hydrogen sulfide by boiling, sodium carbonate, which had been shown to be low in iron, was added. The precipitate of calcium carbonate was washed thoroughly and dried.

The phytic acid used was prepared from crude calcium phytate<sup>6</sup> according to the method of Boutwell (10). It was added to the ration as required in the form of a concentrated solution. This solution contained 108 mg. of phosphorus per cc., of which 8.1 mg. were inorganic. It was also contaminated with 0.90 mg. of ferric iron per cc. Suitable corrections were made for these constituents when incorporated in the diet. The amount of phytic acid fed was approximately 20 times the amount required to form ferric phytate with the iron supplement.

All the rats were given 40  $\gamma$  of  $\beta$ -carotene in Wesson oil by dropper twice weekly. When vitamin D was fed, approximately 50 u.s.p. units (viosterol) were given to each rat daily. The viosterol was dissolved in a small

<sup>5</sup> U.S.P., Merck and Company, Inc.

<sup>6</sup> Obtained from the E. A. Staley Manufacturing Company, Decatur, Illinois.

amount of ether, mixed into the ration, and the ether allowed to evaporate. Inasmuch as vitab is well known to be somewhat deficient in riboflavin for normal growth, 2 mg. of riboflavin were added to each kilo of ration. The constituents of the ration were mixed in 1 or 2 kilo quantities in an aluminum pan. To facilitate its incorporation in the diet, the vitab was diluted with a small quantity of water.

In all the experiments, the iron supplement was corrected for the amount of iron in the basal ration. This amounted to 12  $\gamma$  of iron per gm. when phosphates were the source of phosphorus and 35  $\gamma$  of iron when phytic acid was the source of phosphorus. The total amount of iron given was kept at a fairly low level, namely 0.50 mg. per rat daily, in the first experiment. With this intake it was believed that under the experimental conditions it would be possible to obtain differences in hemoglobin production, or if this failed that differences in the storage of iron could be detected. The iron was added to approximately 3 gm. of the diet each morning in the form of a solution of ferric chloride to which a small amount of hydrochloric acid had been added to prevent precipitation. A solution of 0.10 mg. of copper as copper sulfate was also mixed into the 3 gm. portion of the ration. After this portion of the diet had been consumed, additional ration was fed. The total amount of ration fed was equalized for all rats in the different groups. To avoid errors due to spilling, absorbent paper towels were placed below each cage and any spilled ration was returned to the feed jars each morning. Between feedings, the rations were stored in a refrigerator.

The animals were prepared for the experiments by the Elvehjem and Kemmerer technique (11). At 21 days of age they were weaned and divided into groups, with due precautions to equalize the effect of any variations due to litter origin, weight, sex, and hemoglobin content. Each group contained six rats. The rats were kept in individual suspended cages made of zinc-covered wire, 2 meshes to the inch.

Hemoglobin was determined in 0.02 cc. of blood taken from the tail. The blood was diluted with 5 cc. of distilled water and then with 5 cc. of dilute ammonium hydroxide. The color was read in an Evelyn photoelectric colorimeter with a 540  $m\mu$  filter.

For the analysis of body iron, the rats were killed by etherization and dried in an oven at 104°. The carcasses were then ashed in a muffle furnace at 600° for 2 hours after the addition of 10 cc. of a saturated solution of magnesium nitrate in ethanol and also of 2 gm. of iron-low calcium carbonate to minimize the loss of iron by volatilization. When necessary, the incineration was completed at a low temperature after the addition of a few cc. of iron-low nitric acid. The ash was dissolved in dilute iron-low hydrochloric acid and diluted to volume in redistilled water. Iron deter-

minations were then made on suitable aliquots by the amyl alcohol-thiocyanate colorimetric method as described by Jackson, Klein, and Wilkinson (12) and modified by Nordfeldt (5).

All the rations which were fed in the first series of experiments were checked for their iron, calcium, and phosphorus content. Iron was determined by the method already described. Calcium was determined by the method of Meloche, Clifern, and Griem (13), and phosphorus by the method of Fiske and Subbarow (14).

It is evident from Table I that, with an optimal intake of calcium, the addition of phytic acid, as compared with the addition of inorganic phosphates, resulted in a reduction in the amount of hemoglobin produced. While the effect was not marked, it was significant because the rats were uniform in weight and the reduction occurred consistently. However, phytic acid apparently did not interfere measurably with the storage of iron; the increase in body iron was fully as great when the phosphorus was fed as phytic acid as when it was supplied in an equivalent amount in the inorganic form. When the calcium intake was increased to 4 times the optimal, the effect of phytic acid was the same although both hemoglobin and body iron were depressed by the excessive intake of calcium. This effect of calcium is well known (15-17).

Attention is called to the fact that vitamin D had a beneficial effect on the production of hemoglobin at the lower level of calcium intake. At the higher level of intake no effect was produced. A similar effect of vitamin D on the total amount of body iron was observed, particularly when the diet contained optimal amounts of inorganic calcium and phosphorus. Additional experiments on the beneficial effect of vitamin D on the retention of iron will be published later.

In the second series of experiments, in which whole milk was used as the basal ration, two groups of sixteen rats each were given, respectively, a supplement of 0.20 mg. of iron as ferric ammonium sulfate or ferric phytate. In addition, each rat received daily 0.10 mg. of Cu as  $\text{CuSO}_4$  and 0.10 mg. of Mn as  $\text{MnSO}_4$ . The supplements were fed in a few cc. of milk in the morning. Additional milk was then fed *ad libitum*.

The rats used were 21 days of age and weighed approximately 37 gm. They were distributed between the groups, as in the first series, to equalize variations due to sex, weight, litter origin, and hemoglobin content.

The ferric phytate was prepared from phytic acid by the method of McCance and Widdowson (1) and was fed as a stable suspension in redistilled water. The rats were weighed and hemoglobins determined weekly during the 5 week feeding period.

The results (Table II) show that the production of hemoglobin was consistently less with ferric phytate than with ferric ammonium sulfate. As

TABLE I

*Effect of Dietary Phytic Acid on Hemoglobin and Body Iron*

All rats received an optimal intake of phosphorus (0.25 per cent of the ration), 0.50 mg. of iron, and 0.10 mg. of copper daily except for the last 5 days, when the iron supplements were discontinued. Each group consisted of six rats. The average iron content of the rats at the start of the experiment approximated 0.80 mg. per rat as determined on forty-four rats of the same origin, size, sex, and weight.

Group No.	Vitamin D, U.S.P. units daily	Source of phosphorus	Final body weight	Hemoglobin per 100 cc. blood			Body iron	
				Initial	Final	Increase	Total*	Increase
Optimal calcium								
1		Inorganic	gm.	gm.	gm.	gm.	mg.	mg.
2		Phytic acid	76	8.38	15.24	6.86	4.84	3.39
3	50	Inorganic	75	9.06	14.10	5.04	5.19	3.74
4	50	Inorganic	78	8.42	16.37	7.95	6.03	4.58
		Phytic acid	77	9.07	16.17	7.10	5.52	4.07
4 times optimal calcium								
5		Inorganic	74	8.84	14.75	5.91	3.42	1.97
6		Phytic acid	72	8.68	13.64	4.96	3.60	2.15
7	50	Inorganic	74	9.33	15.04	5.71	3.34	1.89
8	50	Phytic acid	72	9.31	14.00	4.78	3.51	2.06

\* This represents the total iron content of the animals. Analysis of the contents of the digestive tracts of twenty-one rats similarly fed revealed that they contained an average of 0.65 mg. of iron.

TABLE II

*Availability of Iron of Ferric Phytate for Hemoglobin Production*

Each rat received 0.20 mg. of Fe, 0.10 mg. of Cu, and 0.10 mg. of Mn daily during the 5 week feeding period. There were sixteen rats in each group. The basal diet was whole milk.

Group No.	Source of iron*	Hemoglobin						
		Initial	1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	Total increase
		gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	Ferric ammonium sulfate	7.59	10.88	11.03	12.19	12.71	12.97	5.38
2	" phytate	7.78	10.46	10.65	10.94	11.38	12.10	4.32

\* In a control group of five rats on milk plus copper and manganese only, the average level of hemoglobin dropped from 11.03 to 3.82 gm. in 5 weeks.

in the first experiment, these data are significant because of the large number of animals and because the average increase in weight was practically identical for each group. It should be emphasized that the iron of ferric

phytate is fairly available because, as is well known, rats become severely anemic when maintained on whole milk supplemented with copper and manganese, whereas our animals with an additional supplement of ferric phytate increased their hemoglobin content steadily throughout the experiment.

We acknowledge our indebtedness to Mr. Rudolf Bunkfeldt for the supply of phytic acid and to Mr. C. E. Zehner for the supply of cow's milk.

#### SUMMARY

With a "synthetic" ration furnishing a limited amount of iron as ferric chloride and an amount of calcium optimal for growth and calcification, slightly less hemoglobin was produced in rats when the optimal amount of phosphorus was supplied as phytic acid rather than as a mixture of the mono- and dipotassium phosphates. An excess of calcium reduced the formation of hemoglobin and the storage of iron. Vitamin D improved the storage of iron and the formation of hemoglobin when the diet contained optimal amounts of calcium and phosphorus. However, no such effect was observed when the diet contained an excess of calcium. With milk supplemented with copper and manganese as the basal ration, a limited amount of iron as ferric phytate produced 19 per cent less hemoglobin than an equal amount of iron in the form of ferric ammonium sulfate.

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# THE EFFECT OF DIETARY CALCIUM, PHOSPHORUS, AND VITAMIN D ON THE UTILIZATION OF IRON

## II. THE EFFECT OF VITAMIN D ON BODY IRON AND HEMOGLOBIN PRODUCTION\*

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In recent investigations in this laboratory (1), it was found that vitamin D produced a marked increase in the storage of iron. A review of the literature relating to this effect yields little evidence either supporting or contradicting this observation. Shelling and Josephs (2) noted an improvement in the retention of iron in rats with the feeding of viosterol. However, as the authors state, the effect was no more than suggestive. Later, Josephs (3) found that viosterol increased the retention of iron in two out of three infants under observation. Porter (4), on the other hand, was unable to demonstrate any increase in the retention of iron in pre-school children with the replacement of plain farina in the diet with farina which had been fortified with irradiated yeast. It should be noted, however, that her basal diet contained an appreciable amount of vitamin D (40 U.S.P. units per child daily) and, therefore, that this experiment was not directly comparable with that of Josephs.

With regard to the effect of vitamin D on hematopoiesis, Day and Stein (5) observed a mild anemia and polycythemia in rats on a diet containing either normal levels of calcium, but three times the normal amount of phosphorus, or normal amounts of phosphorus, but only one-fifth the normal quantity of calcium. The addition of vitamin D to such rations resulted in somewhat higher hemoglobin values.

Maughan (6) reported the occurrence of an anemia in rachitic chicks which was curable with ultraviolet light. McDonough and Borgen (7), on the other hand, were not able to find any difference in the amount either of hemoglobin or of liver iron of rachitic chicks as compared with normal chicks.

Seyderhelm, Tammann, and Baumann (8), in their experiments with dogs, produced an anemia by diverting the flow of bile to the urinary tract. They found that this anemia could be prevented or cured with bile acids and also with light-activated ergosterol. Further study of this problem

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by Takasu (9) gave similar results. Takasu suggested that the combined action of bile acids and vitamin D was essential for normal hematopoiesis under his experimental conditions.

An additional experiment with a possible bearing on this subject is that of Crowley and Taylor (10). These investigators found that iron-resistant anemia of school children with latent rickets was improved only by the combined feeding of iron and vitamin D. However, Hauss (11), in his review of the literature on the anemia of rickets, cited several reports in which vitamin D, as cod liver oil or ultraviolet irradiation, had no effect on a low level of hemoglobin.

Obviously, additional data which would throw further light on the influence of vitamin D on the utilization of dietary iron are needed. For this reason the following investigations were undertaken.

#### EXPERIMENTAL

The experiments were carried out with young rats and, with certain exceptions, which will be pointed out later, essentially the same ration, technique, and criteria as those which were employed in a previous experiment (1). The iron-low "synthetic" ration contained calcium carbonate and an equimolecular mixture of mono- and dipotassium phosphate to give it a calcium content of 0.35 per cent and a phosphorus content of 0.25 per cent. These amounts had been found to be optimal for growth and calcification. Each rat was given daily a solution of 0.10 mg. of Cu as  $\text{CuSO}_4$  and either 0.10 or 0.50 mg. of Fe as  $\text{FeCl}_3$  mixed daily into a portion of the ration. In each experiment, some of the rats were given 50 u.s.p. units of vitamin D daily as viosterol or as percomorph oil.

Each experimental group was composed of eight or nine rats. The duration of the feeding period was 4 or 6 weeks. Hemoglobin determinations were made weekly or at the beginning and at the end of the experiments. When the experiments were terminated, the animals were killed by decapitation and analyzed for iron after the removal of the digestive tracts. When individual organs were analyzed for iron, they were washed thoroughly with isotonic saline solution before incineration. The blood obtained by these washings together with the blood drawn at decapitation was incinerated with the carcass.

Although a number of separate experiments were run, only two of them will be reported in detail, as the results were similar in all of them. In the first one to be reported, the rats were given 0.50 mg. of iron daily. The consumption of food was controlled by paired feeding; *i.e.*, for each rat receiving vitamin D, one control rat not receiving vitamin D was given the same amount of ration day by day. This technique served to equalize growth and thereby facilitated comparisons of the amount of hemoglobin synthesized and the relative abundance of iron reserves.

Table I gives the results of this experiment. It shows that all of the rats which received vitamin D had a slightly higher hemoglobin level than those which did not receive this supplement. This confirms the results obtained previously (1). While the difference was not large, viz. 1.07 gm., the consistency with which this difference occurred makes it significant. Furthermore, attention is called to the fact that all of the rats which received vitamin D contained appreciably more iron than their paired feeding mates. Only a very small part of this difference was due to the liver differential.

TABLE I

*Effect of Vitamin D on Body Iron, Liver Iron, and Hemoglobin*

The duration of the experiment was 4 weeks. Each rat received 0.50 mg. of iron, and 0.10 mg. of copper daily.

Group 1. No vitamin D							Group 2. 50 units vitamin D per rat daily as viosterol						
Rat No.	Body weight		Hb per 100 cc. blood		Liver Fe	Increase in body Fe*	Rat No.	Body weight		Hb per 100 cc. blood		Liver Fe	Increase in body Fe*
	Initial	Increase	Initial	Increase				Initial	Increase	Initial	Increase		
	gm.	gm.	gm.	gm.	mg.	mg.		gm.	gm.	gm.	gm.	mg.	mg.
750	42	70	7.66	5.92	0.30	1.75	753	43	66	7.35	8.13	0.37	2.38
751	41	49	6.87	8.50	0.29	2.11	761	42	52	7.35	8.87	0.31	2.53
752	40	47	7.57	8.22	0.28	1.86	759	44	46	6.50	9.60	0.30	2.38
753	42	69	6.94	7.61	0.32	2.35	760	40	70	7.16	8.09	0.35	2.72
754	40	72	8.26	5.90	0.38	2.16	762	42	62	8.63	6.04	0.50	2.53
755	44	86	7.28	5.95	0.32	2.79	765	43	80	7.52	6.83	0.34	2.96
756	44	65	7.66	6.50	0.32	2.42	764	44	76	7.94	7.74	0.43	3.09
757	45	72	8.26	5.24	0.36	2.76	763	43	76	7.80	7.45	0.33	3.09
Average...	42	66	7.58	6.71	0.33	2.27		42	67	7.53	7.83	0.37	2.71

\*Includes liver iron.

In this connection, an observation concerning the effect of vitamin D on the size of the liver is of interest. Although vitamin D had no influence on body weight, it increased both the average wet and dry weight of the liver approximately 20 per cent. An inspection of similar data obtained in another experiment of the present series revealed the same effect. However, there was no relationship between the size of the liver and the amount of iron contained therein. Contrary to this observation, vitamin D had no effect on the dry weight of the spleen nor on the amount of iron present.

Because vitamin D increased the quantity of body iron, it was believed

desirable to determine its effect on body copper. The determinations were made by the method of Coulson (12). Apparently, it had no effect; the average amount of body copper per rat was found to be 0.96 mg. for the rats which received vitamin D as well as for those which did not.

In the next series of experiments, certain modifications were introduced to make the test more critical. Twenty-seven 21 day-old rats were given

TABLE II

*Effect of Vitamin D on Hemoglobin Production*

The duration of the experiment was 6 weeks. There were nine rats in each group. Each rat received 0.10 mg. of iron and 0.10 mg. of copper daily.

Group No.	Body weight		Hemoglobin per 100 cc. blood	
	Initial	Increase	Initial	Increase
	gm.	gm.	gm.	gm.
1. No vitamin D.....	83	105	4.86	8.60
2. Vitamin D as viosterol*.....	83	105	5.00	8.90†
3. " " " percomorph oil*.....	83	109	4.98	9.91‡

\* 50 U.S.P. units were given to each rat daily.

† In comparison with Group 1, two rats had a lesser increase, one had no increase, and six had a greater increase in hemoglobin.

‡ In comparison with Group 1, two rats had a lesser increase and seven had a greater increase in hemoglobin.

TABLE III

*Hemoglobin Values As Determined Week by Week on Rats of Table II*

Wks. on experiment	Group 1. No vitamin D	Group 2. 50 units daily as viosterol	Group 3. 50 units daily as percomorph oil
	gm.	gm.	gm.
0	4.86	5.00	4.98
1	6.92	7.62	7.32
2	8.63	9.88	9.15
3	9.44	10.92	10.06
4	10.43	11.60	11.46
5	12.87	13.28	13.63
6	13.46	13.90	14.89

iron-low cow's milk for 3 weeks until the hemoglobin values had decreased to approximately 5.00 gm. Eighteen of the rats were then given the "synthetic" ration with the addition of 50 U.S.P. units of vitamin D per rat daily as viosterol or percomorph oil; nine were kept as controls. All rats were given 0.10 mg. of iron daily. Hemoglobin determinations were made weekly until the values had returned to normal.

The data indicate (Table II) that vitamin D increased the production of hemoglobin only slightly, but consistently. Of the eighteen rats given vitamin D, thirteen, or 72 per cent, had higher hemoglobin values than their paired feeding mates. In the case of viosterol (Group 2), the difference was very slight; the average increase in hemoglobin was 8.90 gm. as compared with 8.60 gm. in the control animals. However, at the end of the experiment, six of the nine rats on viosterol had more hemoglobin than their controls. Furthermore, these differences were consistent week by week (Table III).

Percomorph oil had a similar beneficial action on the synthesis of hemoglobin (Table II). At the end of the experiment, seven out of nine rats receiving this supplement contained more hemoglobin than their controls, the average values being 14.89 and 13.46 gm., respectively. As with viosterol, the effect of percomorph oil prevailed throughout the entire time of the experiment (Table III).

#### SUMMARY

When vitamin D was added to a "synthetic" ration optimal for growth in its content of phosphorus and calcium and of known iron content, there resulted an increase in hemoglobin and especially in the total amount of body iron in rats. Although vitamin D had no effect on body weight, it produced a marked increase in the weight of the liver. In contrast to the beneficial effect on the storage of iron, vitamin D had no effect on the storage of copper.

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# THE EFFECT OF DIETARY CALCIUM, PHOSPHORUS, AND VITAMIN D ON THE UTILIZATION OF IRON

## III. THE RELATION OF RICKETS TO ANEMIA\*

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Anemia has been reported in rachitic infants and children by a large number of clinicians. However, there is little agreement as to its etiology. This state of affairs is well summarized in a quotation from Hess (1). "It is probably true that severe rickets is generally accompanied by anemia. But whether this alteration is to be attributed to the rickets or to the malnutrition occasioned by associated errors of hygiene and diet remains to be determined."

Typical of the contrasting opinions on this subject are the report of McDonough and Borgen (2) and that of Crowley and Taylor (3). The former investigators believe that there is no relationship between rickets and anemia. They found that the feeding of iron salts to rachitic infants with subnormal hemoglobin values raised these figures to normal. They further observed that the administration of vitamin D did not increase the level of hemoglobin in the anemia of uncomplicated rickets. Crowley and Taylor (3), on the other hand, found that only the combined feeding of iron and vitamin D increased the level of hemoglobin in eighteen children with latent rickets.

A similar lack of agreement is evident in the data presented by investigators who have studied this problem with the rat and also with the chick. Happ (4) produced rickets in rats by feeding grain rations which were low in calcium. Anemia developed in seven out of fifteen rats within 3 to 8 months. In addition, the livers and spleens were very pale, indicating that the iron stores were reduced below normal. Sure and Kik (5) and also Hauss (6) were not able to find any difference between the blood picture of rachitic and that of normal rats.

Using the chick as the experimental animal, Maughan (7) studied the effect of ultraviolet irradiation on the amount of hemoglobin when a rachitogenic, vitamin D-low ration was fed. He found that hemoglobin values were markedly lower in the rachitic chicks than in the irradiated birds. He further reported that the cure of rickets was accompanied by an in-

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crease in hemoglobin to the normal figures. Contrary to these results, McDonough and Borgen (2) were not able to detect any difference in the level of hemoglobin between normal and rachitic chicks. In addition, the amount of iron per gm. of dried liver was approximately the same whether or not cod liver oil had been included in the diet.

All of these experiments had one or more experimental defects. For example, the diets were composed of naturally occurring foods, the phosphorus of the diet was furnished in various degrees of availability, the source or amount of dietary iron was not controlled, the type of rickets induced was not ascertained, and the severity of the rickets was not indicated. These circumstances made it desirable to carry out additional experiments with a diet compounded of purified food constituents in which rickets was produced by definite deficiencies or imbalances.

#### EXPERIMENTAL

Except for the omission of 219.44 parts of calcium carbonate from the salt mixture (8), the iron-low "synthetic" ration previously described was fed. The desired proportions of calcium and phosphorus were attained by the addition of calcium as the carbonate and phosphorus as an equimolecular mixture of mono- and dipotassium phosphates. As before, the diet was considered to be optimal with respect to its content of calcium and phosphorus when it contained 0.35 and 0.25 per cent of these, respectively. These proportions were varied from one-eighth to 4 times optimal. Each rat was given 0.10 mg. of copper as the sulfate together with a certain amount of iron as ferric chloride in redistilled water mixed into a small portion of the ration daily.

At the end of the feeding period, the rats were killed with ether and both femurs and the distal ends of the radius and ulna were dissected out. The femurs were extracted with 95 per cent ethanol for 72 hours and then dried in a muffle furnace at 650° until free from carbon. The ends of the radii and ulnae were split longitudinally with a scalpel, stained with silver nitrate, and inspected for the width of the metaphyses.

The experiments were carried out in two series, the second series differing from the first primarily in that some of the rations were supplemented with vitamin D and the consumption of ration was equalized between individuals of different groups. In addition, in the second series the carcasses of the rats were analyzed for iron.

The results of the first series (Table I) show clearly that the low phosphorus and the low calcium diets produced rickets. However, the high calcium diet failed to do so. This was undoubtedly due to the fact that the phosphorus was present in the inorganic form. It has frequently been noted that a high calcium ration will produce severe rickets, but this is

TABLE I

*Effect of Variable Amounts of Dietary Calcium and Phosphorus on Hemoglobin Formation*

Three males and three females, 21 days of age, were used in each group. Each rat was given 0.20 mg. of iron and 0.10 mg. of copper daily for 5 weeks.

Group No.	Body weight		Hemoglobin per 100 cc. blood				Bone ash	Metaphysis
	Initial	In-crease	Initial	Increase after				
				1 wk.	3 wks.	5 wks.		
	gm.	gm.	gm.	gm.	gm.	gm.	per cent	
1. Optimal Ca, $\frac{1}{2}$ optimal P. . . .	34	41	8.22	2.53	4.19	5.44	21.7	Severely rachitic
2. Optimal Ca, $\frac{1}{4}$ optimal P. . . .	33	22	8.43	2.45	3.18	7.08	19.9	" "
3. Optimal Ca, 4 times optimal P. . . . .	32	52	8.42	3.84	4.37	6.26	46.4	Normal
4. $\frac{1}{2}$ optimal Ca, optimal P. . . . .	31	37	8.50	3.07	3.74	5.82	38.3	Mildly rachitic
5. 4 times optimal Ca, optimal P. . . . .	40	49	7.01	0.07	1.90	1.80	48.4	Normal
6. Optimal Ca, optimal P. . . . .	33	72	8.67	3.18	2.68	4.04	45.8	"

TABLE II

*Effect of Low Intake of Dietary Calcium and Phosphorus on Body Iron and Hemoglobin Formation*

Three males and three females, 21 days of age, were used in each group. Each rat was given 0.50 mg. of iron and 0.10 mg. of copper for the first 23 days of the 28 day feeding period.

Group No.	Viosterol per rat daily, u.s.r. units of vitamin D	Body weight		Hb per 100 cc. blood		Increase in body iron*		Bone ash	Metaphysis
		Initial	Increase	Initial	Increase	Total	Per gm. body weight		
		gm.	gm.	gm.	gm.	mg.	mg.	per cent	
1. Optimal Ca, optimal P...	0	37	39	8.38	6.86	3.39	0.049	48.8	Normal
2. Optimal Ca, optimal P ..	50	35	43	8.42	7.95	4.58	0.059	50.6	"
3. Optimal Ca, $\frac{1}{2}$ optimal P.	0	37	38	8.32	6.91	3.04	0.041	29.2	Severely rachitic
4. Optimal Ca, $\frac{1}{4}$ optimal P.	50	37	38	8.32	7.69	3.33	0.044	38.1	Mildly rachitic
5. Optimal P, $\frac{1}{2}$ optimal Ca.	0	36	16	8.24	6.54	2.85	0.055	38.8	" "
6. Optimal P, $\frac{1}{4}$ optimal Ca.	50	37	24	8.30	7.41	3.34	0.055	36.3	" "

\* This does not include the iron of the metaphyseal specimens.



brought about with facility only when the phosphorus is present in such a form that it forms difficultly soluble salts, as, for example, when the phosphorus is present as phytic acid (9).

With regard to hematopoiesis, it is seen that neither low phosphorus nor low calcium rickets produced anemia. On the other hand, a pronounced anemia occurred in the high calcium group. This action of calcium has been reported by several investigators (8, 10-14).

In addition, attention is called to the fact that a phosphorus intake high in relation to the rat's requirement or high in relation to the intake of calcium did not affect the production of hemoglobin, even though a mild rickets resulted in the latter instance. These results are contrary to those obtained by Day and Stein under similar experimental conditions (15). However, our animals grew less in relation to their controls than those of the aforementioned investigators, so that, even with a reduction in the amount of iron assimilated, the requirement for hemoglobin synthesis may have been met.

In the second series of experiments in which the effect of vitamin D was determined, particular care was taken to select the rats for uniformity of weight and hemoglobin content. Furthermore, the intake of food was equalized for all of the rats until the 4th day of the experiment, when the groups receiving one-eighth optimal calcium reduced their food intake to an excessive degree. From that time on, they were treated as a separate series. However, the iron intake of all the rats was kept constant throughout the experiment.

Results of this series (Table II) show that with a low intake of calcium and of phosphorus rickets was produced but hemoglobin synthesis again was not affected. However, as observed previously (8, 15, 16), the addition of vitamin D resulted in all instances in an increase in hemoglobin values over the levels contained in the control animals.

Although there was less iron per gm. of body weight in the low phosphorus-fed rats than in the controls on optimal phosphorus, the poorer retention of iron was not due to rickets *per se*. This is proved by the fact that, although vitamin D induced marked healing, there was little change in the amount of body iron. It is possible that the somewhat poorer retention of iron in these groups was due to the relative excess of calcium over phosphorus in the diet. The amount of iron per gm. of body weight was as great in the rachitic animals on suboptimal calcium as in the non-rachitic rats on optimal calcium and phosphorus.

#### CONCLUSION

Low calcium and low phosphorus rickets did not reduce the amount of body iron nor the rate of hemoglobin synthesis in the rat.

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# PURIFICATION OF THE GROWTH HORMONE OF THE ANTERIOR PITUITARY\*

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The present communication describes a procedure of established dependability for the preparation and purification of the growth hormone of the anterior pituitary. The procedure is a final result of many modifications in method attempted during the last few years.

## *Methods of Assay*

In the course of fractionation procedures it is important to follow the progress of purification with reliable determinations of hormonal potencies of the fractions obtained. The method used here for the assay of growth hormone was described recently (1). It is based on the body weight increase of rats injected daily for periods of 10 to 20 days. Depending on the character of the particular preparation to be tested, and on the accuracy required in a given case, either normal female rats whose weight had reached a plateau were used (injection periods, 15 and 20 days), or hypophysectomized immature female rats (injection periods, 10 and 15 days). Hypophysectomized animals were employed in some instances for a second and even for a third course of injections, although it was realized that the accuracy was less in these cases. Growth hormone units, GU, were defined, as already described, for each type of test animal and for each test period, and growth hormone potencies were expressed as growth hormone units per mg. of hormone preparation, [GU], special references being made to the particular experimental conditions of a given case.

In order to be able to compare results of assays conducted with different types of test animals, or with different injection periods, conversion factors were determined which permit approximate expression of the potency of a growth hormone preparation in terms of any one of the assay methods enumerated. These conversion factors were derived from ratios of average values for growth hormone potencies which had been established empirically (Table I). A growth hormone preparation which would cause exactly 10 gm. of body weight increase in 10 days, when given to

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hypophysectomized rats at a level of 100  $\gamma$  daily, would produce body weight increases and, therefore, have growth hormone potencies in other tests as indicated in Table I.

Crude pituitary extracts contain, besides growth hormone, various other active principles, five of which are generally accepted today as separate entities—the follicle-stimulating (FSH), interstitial cell-stimulating (ICSH) lactogenic, thyrotropic, and adrenocorticotrophic (ACTH) hormones. Therefore, it is not sufficient to follow purification by measuring growth hormone potency per mg. alone. As a further criterion for purity, it is desirable to determine that there is a quantitative reduction in the other “contaminating” hormones. For this purpose, growth hormone prepara-

TABLE I  
*Correlation of Methods of Growth Hormone Assay*

Type of test rat	Injection period	Daily dose	Body weight increase per day	Potency*	Conversion factor
	days	mg.	gm.	units per mg.	
Hypophysectomized	10	0.1	1.00	10.0 [GU] <sub>HR</sub> <sup>10</sup>	1.00
“	15	0.1	0.99†	9.9 [GU] <sub>HR</sub> <sup>15</sup>	0.99
“	10	0.1	0.80‡	6.1 [GU] <sub>UHR</sub> <sup>10</sup>	0.61
previously injected					
Normal (weight plateau)	15	1.0	1.65§	0.6 [GU] <sub>NR</sub> <sup>15</sup>	0.06

\* [GU] = growth hormone units per mg. of hormone preparation. Explanation of indices: 10 = 10 day test; 15 = 15 day test; HR = hypophysectomized rats; UHR = hypophysectomized rats, previously injected; NR = normal rats at a weight plateau.

† Mean value, derived from 349 test animals.

‡ Mean value, derived from 225 test animals.

§ Mean value, derived from 412 test animals.

tions were injected, at relatively high levels, into immature pigeons to assay for lactogenic hormone (response of crop sac), and into hypophysectomized rats for determination of the other four pituitary hormones enumerated (responses of ovaries, thyroids, and adrenals).

### *Method of Purification*

The method used at present for purification of the anterior pituitary growth hormone consists of a series of five steps: desiccation of beef anterior pituitary lobes with acetone, extraction with calcium hydroxide, precipitation with ammonium sulfate, treatment with cysteine, and fractionation by pH variation. The entire procedure is carried out in a cold room at 2–5°, with a few exceptions specifically mentioned.

1. *Preparation of Acetone Powder*—1 kilo of anterior lobes of beef

pituitaries (which are stored frozen) is ground at room temperature in a mechanical meat grinder to a fine mash (with addition of 250 cc. of water for rinsing purposes). The material is then refrozen and reground. The ground material is added to 6 liters of acetone at  $2-5^{\circ}$ , and the mixture is stirred for 2 hours and then allowed to settle overnight at  $-10^{\circ}$ . The supernatant fluid is separated by syphoning, the rest of the acetone is removed by quick filtration, and the procedure repeated. Supernatant fluids are discarded. The filter cake is dried quickly between filter papers, and is then spread on the bottom of a large desiccator (no drying agent) which is connected with the house vacuum, for 3 days at  $2-5^{\circ}$ , and finally 1 day at room temperature. The resulting product, designated "acetone powder," has a pale pinkish tinge. When kept dry, it can be stored at  $-10^{\circ}$  for at least a year without loss of growth hormone potency. Yield, approximately 230 gm.

2. *Alkaline Extraction with Calcium Hydroxide*—The acetone powder (230 gm.) is ground and then suspended in 8 liters of cold water. A suspension of calcium hydroxide (30 gm. of CaO per liter) is then added with stirring until the pH reaches 11.2 to 11.5 (glass electrode). Approximately 320 cc. are necessary. Stirring is continued over a period of 24 hours. Then, 50 cc. of a calcium chloride solution (100 gm. of  $\text{CaCl}_2$  per liter of water) are added and carbon dioxide gas is passed through the mixture with stirring to bring the pH to 8.5. The mixture is allowed to stand for 1 to 2 days for sedimentation of the insoluble material. The supernatant fluid is then removed by syphoning and the residue filtered overnight through Filter Cel (Buchner funnel). The filter cake is washed with about 1 liter of water. The dark brown supernatant fluid and filtrate combined are called the "calcium hydroxide extract." This extract can be stored in the frozen state, but, usually, step (3) is carried out immediately. Yield, approximately 62 gm.

3. *Precipitation with Ammonium Sulfate*—Solid ammonium sulfate is added slowly with stirring to the calcium hydroxide extract until the solution is 0.5 saturated. Stirring is continued for 1 hour; the mixture is then allowed to stand for 1 to 2 days for sedimentation of the precipitate. The supernatant liquid is removed by syphoning, and the rest of the mixture is filtered through hardened filter paper (Buchner funnel), with only a low vacuum in the initial stages. The supernatant fluid and filtrate are discarded. The precipitate is redissolved at pH 9 to 9.5 in approximately one-fourth of the original volume, insoluble material is removed by centrifugation, and the precipitation with ammonium sulfate is repeated, this time at 0.45 saturation, preferably by adding saturated ammonium sulfate solution. The precipitate is suspended in as little water as possible, and dialyzed through cellophane against distilled water in a rocking dialyzer.

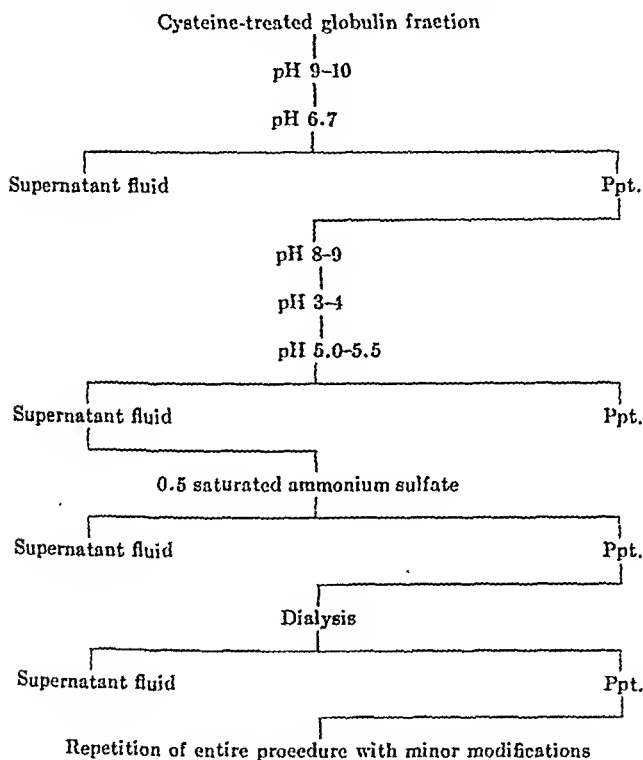
After complete removal of inorganic salts the mixture is centrifuged. If sedimentation is not satisfactory, it can be improved by adjusting the pH of the mixture to 6.5 with a few drops of 0.1 N HCl. The sediment, which contains practically all the growth hormone, is dried in a vacuum desiccator over anhydrous sodium hydroxide or phosphorus pentoxide at room temperature. The supernatant liquid is discarded. The dry product which usually contains some gray-brown pigment is designated the "globulin fraction." Yield, approximately 16.3 gm.

4. *Treatment with Cysteine*—This step is carried out at room temperature. The hormone and cysteine are first dissolved separately, both at relatively high concentrations. For solution of the globulin fraction, 16.3 gm. are suspended in 100 cc. of water, and N NaOH is added to bring the pH to 9 to 10. After being homogenized as far as possible, the mixture is centrifuged, and the residue dissolved with an additional 100 cc. of water and N NaOH. A cysteine solution is then made by suspending 20 gm. of cysteine hydrochloride in 40 cc. of water and adding slowly the necessary quantity of N NaOH (cooling with ice water). The hydrogen ion concentration of both solutions is then adjusted to pH 8 to 8.5, and the cysteine solution added to the hormone solution. The resulting mixture is kept in a tightly stoppered flask for 24 hours. A pinkish gel is formed as a result of the reaction. The mixture is then diluted with water to 1 liter, is centrifuged, and the precipitate is reextracted first with 500 cc., then with 300 cc. of water. The residue is discarded. When the combined supernatant fluids are kept at 3–5° overnight in contact with air, some cystine is sedimented which can be removed by decanting and centrifuging. An equal volume of saturated ammonium sulfate solution is then added. The precipitate formed is separated from the supernatant fluid by centrifugation, is suspended in as little water as possible, and dialyzed. During dialysis a sediment is formed which contains practically all the growth hormone, and in addition, frequently some cystine which is removed during the following step. The clear supernatant fluid is removed by centrifugation and discarded, and the precipitate dried in a desiccator. The dry product, designated the "cysteine-treated globulin fraction" has a grayish tinge. Yield, approximately 6.7 gm.

5. *Fractionation by pH Variation*—This part of the procedure is outlined in the scheme shown in Diagram 1. It consists in principle of an "isoelectric" precipitation at pH 6.5 to 6.7, approached from the alkaline side, at which most of the growth hormone is precipitated, followed by a precipitation at pH 5.0 to 5.5 approached from an acid medium, under which conditions most of the growth hormone activity remains in the supernatant fluid and appreciable quantities of growth-inactive substances are precipitated. The material soluble at pH 5 to 5.5 is then concentrated by ammonium sulfate precipitation.

The procedure is conducted in detail as follows: The cysteine-treated globulin fraction is suspended in water (6.7 gm. in about 100 cc.) and dissolved by adding the necessary amount of 0.1  $N$  NaOH. The cysteine-treated preparation usually is not completely soluble. The insoluble

DIAGRAM 1

*Scheme of Fractionation by pH Variation\**

\* Repetitions of individual steps are not indicated.

part is removed by centrifugation and is, after reextraction at pH 9 to 10, discarded. The combined supernatant fluids are diluted so that the protein concentration is 1 to 1.5 per cent. Hydrochloric acid (0.1  $N$ ) is then added slowly, until pH 6.7 is reached. Appreciable precipitation occurs. The mixture is allowed to stand a few hours and then centrifuged. Most of the growth hormone potency is contained in the sediment. The



supernatant fluid is discarded. The sediment is redissolved with as little 0.1 N NaOH as possible (protein concentration approximately 0.5 per cent), and then acidified slowly with 0.1 N HCl, passing through the isoelectric range, until the precipitate formed is redissolved (at pH 3 to 4; never below 3). The solution is immediately rendered less acid, 0.1 N NaOH being added slowly as pH 5.0 to 5.2 is approached. Appreciable precipitation occurs. The mixture is centrifuged after  $\frac{1}{2}$  to 1 hour, and the supernatant fluid, which contains most of the growth hormone potency, is neutralized. In order to repeat this step, the precipitate is redissolved with 0.1 N NaOH (protein concentration approximately 0.5 per cent), again acidified to pH 3 to 4, and the solution again immediately made less acid, this time being adjusted to pH 5.3 to 5.5. The precipitate formed is removed by centrifugation and discarded. The combined supernatant fluids are neutralized, and an equal volume of saturated ammonium sulfate solution is added. The precipitate formed, which contains practically the entire growth hormone activity, is suspended in as little water as possible and dialyzed.

After completion of the dialysis of the ammonium sulfate precipitate, the entire procedure is repeated with minor modifications. From this stage, subsidiary fractions are saved, since they may contain appreciable amounts of growth hormone. The repetition of the first step of the procedure, precipitation at pH 6.7, is conducted at a slightly higher protein concentration (1.5 to 2 per cent). The precipitate formed at pH 6.7 is redissolved with 0.1 N NaOH, acidified to pH 3 to 4, and then immediately rendered less acid as before, except that this time, 0.1 N NaOH is added only to approximately pH 4.5. The next step, precipitation of inactive substances, is this time conducted at a slightly higher pH according to McMeekin's technique of rotating dialysis (2). A cellophane bag is filled with the solution and rotated in 0.02 M phosphate buffer at pH 5.8. After completion of the dialysis the sediment formed is removed by centrifugation, and the supernatant fluid is again made half saturated with ammonium sulfate. The precipitate is sedimented by centrifugation, suspended in as little water as possible (complete solution is not necessary), and dialyzed. After complete removal of electrolytes, the whole contents of the bag are collected, without separating the sediment from the supernatant fluid, since both fractions contain growth hormone and since the potencies of the two fractions are usually not much different. They represent the final product of the purification and are designated "purified growth hormone." Yield, approximately 500 mg.

#### DISCUSSION

*Fractionation Procedure*—Beef anterior pituitary lobes only were used as starting material, since a comparison of the growth hormone activities

extractable under identical conditions from anterior lobes of beef pituitaries and from whole pituitaries of hog and of sheep had indicated that the most potent extract and the highest yield in activity were to be obtained from beef glands (potencies and yields of the extracts both decreasing in the order, beef, hog, sheep).

Step (1) of the purification procedure, treatment of the ground gland tissue with acetone in the cold, was adopted, since it was observed that handling of the material was markedly facilitated, without any loss in potency; insoluble material settled more readily, facilitating removal of supernatant fluids by syphoning and filtration. In this way, centrifugations of large quantities of liquids, as used formerly, were replaced by procedures which required only a fraction of the time. An additional advantage was the fact that filtration allows a more complete separation of supernatant fluids from precipitates than ordinary centrifugation. These improvements concern all steps in which large quantities of fluid are handled.

Step (2), the calcium hydroxide extraction, was carried out as described by Fraenkel-Conrat *et al.* (3). The total unitage recovered was similar to that reported previously, but the yield in gm. was somewhat higher and the potency per mg. correspondingly lower.

Step (3), the preparation of the globulin fraction by precipitation with ammonium sulfate, represents a modification of the former procedure described by Evans *et al.* (4). No attempt was made to remove protein at 0.2 saturated ammonium sulfate, as described formerly, since it was found that appreciable quantities of growth hormone might be lost in this precipitate, without improving the potency of the globulin fraction. Instead of four successive precipitations with ammonium sulfate as recommended previously (4), only two were carried out. Several lots were carried through with even only one ammonium sulfate precipitation, and the resulting globulin fractions were not much different in yield or potency from those obtained by the standard method of two successive precipitations.

Treatment of the globulin fraction with cysteine (step (4)) was carried out essentially as described by Fraenkel-Conrat *et al.* (3), with only minor modifications. The yield was found rather variable. In our experience thioglycolic acid was not found as satisfactory as cysteine for this step in the preparation of growth hormone (3). At similar molar thiol concentrations, an appreciable portion of the growth hormone activity was destroyed; at lower reagent concentrations, biologically active contaminations were not so well removed.

Further purification of the growth hormone was achieved by fractionation by pH variation. In a solution containing a mixture of proteins, successive shifts in pH cause precipitation of different proteins, since iso-

electric points and, therefore, minimum solubilities lie usually at different hydrogen ion concentrations. This makes possible a separation of the various components of the mixture. In such a fractionation it is important to standardize all experimental conditions rigorously, since the precipitation of a protein is influenced by many factors. These include not only the solubility of a given protein in its pure state, as a function of pH, but also the nature and concentration of electrolytes present, and temperature. In a mixture of proteins the nature and concentration of each of the individual proteins present may also influence solubility. It is particularly important, whether, when the hydrogen ion concentration is adjusted, a given pH is approached from the alkaline or acid side, and whether regions are passed during shifts of pH in which other components of the mixture are precipitated, and whether the change in pH is brought about gradually or quickly. These factors play a decisive rôle, since the growth hormone appears to be easily adsorbed on precipitates of other proteins and carried down at hydrogen ion concentrations at which it would be completely soluble when in a state of higher purity. This may explain differences in pH values reported for the isoelectric point of growth hormone by different investigators (3, 5).

Step (5), the fractionation by pH variation, was adopted in accord with these considerations. It consisted essentially in a precipitation at pH 6.7, approached from the alkaline side, in which most of the growth hormone was precipitated, followed by a precipitation at pH 5.2 to 5.8, approached from the acid side, in which most of the growth hormone remained in solution, but other proteins were precipitated (Diagram 1). An ammonium sulfate precipitation followed for concentration of the product.

During dialysis of the final ammonium sulfate precipitate, sedimentation always occurred within the cellophane bag. Usually the precipitate was not separated from the supernatant fluid, since both fractions were found very active, and since the difference in their potencies was small. Only in a few instances were the two fractions separated by centrifugation, and it was observed that the supernatant fluid was more potent when the protein concentration of the contents of the dialysis bag was below 0.7 per cent but that the precipitate was more potent when the protein concentration was above 1.5 per cent.

*Growth Hormone Potency*—Table II summarizes the growth hormone potencies of the products at each step of the fractionation. Individual lots varied considerably; the values shown represent averages from many repetitions of the procedure.

The globulin fraction was approximately 3 times as potent as the calcium hydroxide extract, this potency being somewhat superior to that of

the L fraction as described previously (4), and similar to that reported by Fraenkel-Conrat *et al.* for such fractions (3).

The growth hormone potency of the cysteine-treated globulin fraction was similar to that of the untreated globulin fraction, although it contained markedly fewer contaminants. The cysteine-treated preparations contained somewhat more inert material than those described previously (3).

These impurities (some of protein nature and some cystine) were easily removed in step (5) in which approximately a 5-fold increase in potency was achieved. The best preparations of the final product obtained had a potency of about 200  $[GU]_{HR}^{10}$ ,\* which corresponds to a daily dose of 5  $\gamma$  necessary to produce a body weight increase of 1 gm. per day in hypo-

TABLE II

*Purification of Growth Hormone. Average Yields and Potencies of Products of Individual Steps*

Step of purification	Yield per kilo anterior lobes	Potency of product	Total activity recovered
	gm.	$[GU]_{HR}^{10}$ *	$GU_{HR}^{10}\dagger$
1. Acetone drying.....	230		
2. Calcium hydroxide extraction .....	62	8	500,000
3. Ammonium sulfate precipitation.....	16.3	24	390,000
4. Cysteine treatment.....	6.7	26	170,000
5. pH fractionation.....	0.5	130	65,000

\*  $[GU]_{HR}^{10}$  = growth hormone units per mg. of hormone preparation in a 10 day test in hypophysectomized rats.

†  $GU_{HR}^{10}$  = growth hormone units in a 10 day test in hypophysectomized rats.

physectomized rats. The average growth hormone potency of these preparations was 100 to 150  $[GU]_{HR}^{10}$ .

*Contamination with Other Anterior Pituitary Hormones*—Besides growth hormone potency, freedom from other pituitary hormones was considered an important criterion for purity, and the products of steps (3), (4), and (5) of the procedure were assayed, not only for growth hormone, but also for FSH, ICSH, thyrotropic and lactogenic hormones, and for ACTH. The results are summarized in Tables III and IV, the values representing averages from many lots.

Table III indicates that the globulin fraction contained appreciable

\* The growth hormone potency was determined by means of a standardization curve described recently (1). The slope of this line which was established repeatedly at different stages of the purification of the growth hormone remained practically constant. This slope has not yet been redetermined for the fraction obtained by the last step of the procedure here discussed.

amounts of all other generally "accepted" anterior pituitary hormones, with the exception of FSH. After cysteine treatment, the concentrations of the remaining contaminants were markedly reduced. ICSH and lactogenic hormone were practically completely removed, and the concen-

TABLE III

*Contamination of Growth Hormone Preparations with Other Pituitary Hormones*

Growth hormone preparation	Highest dose level at which preparation was found negative for contaminating hormone*				
	FSH	ICSH	Lactogenic hormone	Thyrotropic hormone	ACTH
	mg.	mg.	mg.	mg.	mg.
Globulin fraction.....	4.5†	1.25	1.0	1.25	2.0
Cysteine-treated globulin fraction.....	10.0†	10.0†	20.0†	5.0	7.5
Purified growth hormone.....	10.0†	10.0†	(20.0)†	10.0†	10.0†

\* The assay used for lactogenic hormone was the systemic crop test in immature pigeons; all other hormones were assayed in hypophysectomized rats.

† Higher levels were not tested.

‡ The value for contamination by lactogenic hormone refers to the cysteine-treated globulin fraction from which purified growth hormone was derived. The latter has not been assayed in pigeons as yet.

TABLE IV

*Contamination of Purified Growth Hormone with Other Pituitary Hormones*

Contamination by	Highest total dose of purified growth hormone found negative for contamination*	Total minimum effective dose of best preparation of contaminating hormone*	Contamination of purified growth hormone
	mg.	mg.	per cent
FSH.....	10.0	0.004	0.05
ICSH.....	10.0	0.008	0.1
Lactogenic hormone.....	(20.0)†	(0.1)	(0.5)
Thyrotropic hormone.....	10.0	0.03	0.3
ACTH.....	10.0	0.05	0.5

\* The assay used for lactogenic hormone was the systemic crop test in immature pigeons; all other hormones were assayed in hypophysectomized rats.

† The value for contamination by lactogenic hormone refers to the cysteine-treated globulin fraction from which the purified growth hormone was derived. The latter has not been assayed in pigeons as yet.

tration of thyrotropic hormone was significantly diminished, in agreement with former observations (3). Contamination of the cysteine-treated preparations with ACTH was smaller than reported previously, a total dose of 7.5 mg. being usually negative for ACTH (4 day test in hypophysectomized rats).

The final product of the purification was so low in physiologically active contaminants that a total dose of 10 mg. did not cause any demonstrable stimulation of ovaries, thyroids, or adrenals in hypophysectomized rats (4 day test, Tables III and IV). Even the cysteine-treated globulin fraction was so free of lactogenic hormone that 20 mg. did not stimulate the crop sac in the immature pigeon (systemic crop test). On the basis of these results and with the values for the minimal effective doses of the best preparations of the other pituitary hormones now available, it is possible to calculate maximum values, expressed in per cent, for the contamination of the growth hormone preparations with the other "accepted" pituitary hormones. In Table IV, these values are given for the purified growth hormone.

*Recovery of Potency*—In the present study the state of purity of the final product (as determined by growth hormone activity per mg. of substance and by freedom from "contaminating" hormones) was considered more important than its yield. Procedures which permitted advance in the purification accordingly were adopted, even if yields were not satisfactory. Approximately 500,000  $\text{GU}_{\text{HR}}^{10}$  (hypophysectomized rat units) were extracted with calcium hydroxide from 1 kilo of beef anterior lobes, and approximately 65,000  $\text{GU}_{\text{HR}}^{10}$  were recovered in the final product (Table II). The actual yield in growth hormone activity is probably better than appears from these values, for the following reason. The products of the initial three steps of the purification contain appreciable amounts of synergistic factors, especially thyrotropic hormone (6). These fractions, therefore, contain actually less growth hormone than is apparent from bioassays. The synergistic factors are removed to a large extent during the purification procedure, and bioassays of the final product give a more accurate measure of the actual growth hormone concentration.

It is unlikely that the relatively low yield is due to destruction of activity during the purification procedure. Only step (4), the cysteine treatment, may cause inactivation of a small part of the hormone. A relatively larger portion of the activity goes into subsidiary fractions from which it can be recovered. As far as recovery of other pituitary hormones is concerned, no attempt was made in this investigation to purify other active principles simultaneously, and, therefore, the method of purification of the growth hormone discussed here should not be compared with procedures in which several of the pituitary hormones are prepared simultaneously (5, 7).

#### *Properties of Purified Growth Hormone Preparations*

The purified growth hormone has all the properties typical for proteins. According to its solubility in ammonium sulfate solutions it would be

classified as a globulin. At earlier stages of the purification procedure, most of the growth hormone activity is precipitated at pH 6.0 to 7.5 in solutions having a low salt content. Upon further purification the hormone appears to become increasingly soluble and does not precipitate completely under these conditions, even at protein concentrations of 1 to 1.5 per cent. The possibility exists that the pure hormone is more soluble than it appears to be in the preparations obtained so far, and that it is easily adsorbed and carried down on other proteins of globulin nature present in the preparations. For this reason, it would hardly seem advisable to classify the growth hormone as a euglobulin, as proposed by others (5), until quantitative solubility measurements have been carried out with purer preparations. Such determinations have as yet not been conducted, since electrophoresis measurements in the Tiselius apparatus<sup>1</sup> have shown that the purified preparations here described still consist of two components, one of which represents approximately 60 to 70 per cent of the total. For the same reason, analytical chemical studies have been postponed.

No deterioration of the purified product was demonstrable when it was kept for a period of a year as a dry powder *in vacuo* at 3-5°. In hormone solutions, it is of course of great importance to inhibit bacterial growth, in order to prevent loss of activity. Storage in the frozen state was found impractical for this purpose, since it caused loss of an appreciable part of the potency of purified preparations. Instead, butyl alcohol was added to 1 to 2 per cent as a bacteriostatic. Such solutions can be injected into rats in 1 cc. quantities without any injurious effects. Stability of the growth hormone in solution depends on many other factors, such as temperature, hydrogen ion concentration, hormone concentration, presence and concentration of other proteins, inorganic salts, oxygen, etc. At 3-5°, neutral solutions were stable for several weeks. At pH 3.0 or below, practically all growth hormone potency was lost within 12 days at this temperature; at pH 4.0, no demonstrable destruction of activity occurred within 6 days, but some potency was lost after a period of 8 days. In alkaline solutions, the hormone appeared to be relatively more stable. The high initial pH of 11.2 to 11.5 of the calcium hydroxide extraction (step (2)) did not have any injurious effects, as far as could be estimated. Heating of a neutral hormone solution (globulin fraction) for 1 hour at 80° destroyed an appreciable portion of the potency, and immersion in boiling water for  $\frac{1}{2}$  hour decreased the activity markedly, without causing any precipitation.

<sup>1</sup> Kindly carried out by Dr. C. H. Li.

## SUMMARY

A procedure is described for the purification of growth hormone from beef anterior pituitary lobes, consisting of the following five steps: desiccation of the gland tissue with acetone, extraction with calcium hydroxide, precipitation with ammonium sulfate, treatment with cysteine, and further fractionation by pH variation.

The final product had a potency of approximately 130 growth hormone units per mg. (hypophysectomized rat units). Compared with the calcium hydroxide extract, this represents approximately a 16-fold increase in potency.

The final product contained less than 0.05 per cent of the follicle-stimulating hormone, less than 0.1 per cent of the interstitial cell-stimulating hormone, less than 0.3 per cent of the thyrotropic hormone, less than 0.5 per cent of the lactogenic hormone, and less than 0.5 per cent of the adrenocorticotrophic hormone.

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# FERRITIN

## II. APOFERRITIN OF HORSE SPLEEN

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(Received for publication, November 3, 1942)

In Paper I of this series (1) it has been shown that ferritin, although crystallizing readily as a cadmium salt, is, in the dissolved state, not a homogeneous protein as judged from its behavior on centrifugation, from the phase rule solubility test, and from a noticeable if small variability in the percentage of Fe, P, and N in various samples. It is the purpose of this paper to describe a procedure by which the iron of ferritin can be removed and an iron-free, colorless protein prepared, which is homogeneous and crystallizes under the same conditions and with the same crystal form as ferritin. This protein will be referred to as apoferritin. The inhomogeneity of ferritin will become understandable from this study.

Crystals of ferritin and apoferritin are shown in Fig. 1.

*Removal of Iron and Formation of Apoferritin*—When the iron of ferritin is reduced from the ferric to the ferrous state in a solution not acid enough to denature the protein (*i.e.* below pH 4) yet acid enough to be compatible with the existence of ferrous ions in solution, the ferrous ion may be combined either with *o*-phenanthroline or with  $\alpha, \alpha'$ -bipyridine to form a tightly bound water-soluble and dialyzable ferrous complex. The reduction can be brought about by sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ , sodium hydrosulfite). The inability of Kuhn, Sørensen, and Birkofer (2) to reduce the iron even with hydrosulfite, except under conditions in which the protein is denatured, may be explained by the fact that the reduction is slow and that, unless the pH of the solution is kept decidedly acid, ferrous ion may not be detectable. For example, when sodium dithionite is added to a ferritin solution in the presence of *o*-phenanthroline at pH 4.6, the pink color of the ferrous complex appears immediately. At pH 5.4 the formation of the complex takes place more slowly, and at pH 7.0 only traces of it are formed. By carrying out the reduction in a dialysis bag at pH 4.6 in the presence of bipyridine or phenanthroline and subsequently dialyzing away the ferrous complex, ferritin can be freed from its iron. Eventually there results a colorless protein solution which, on addition of cadmium sulfate, gives rise almost immediately to colorless crystals of the same shape as the brown ferritin crystals. They show in solution the usual color tests for proteins. If the crystallization is accomplished in a solution from which only part of the iron is removed, the crystals are accordingly more

or less pale brown. It is possible to obtain crystals ranging from deep brown to colorless with an iron content of from 23 per cent to 0 without any variation in the crystal form. Evidently the ability to crystallize as a cadmium salt is a property of the colorless apoferritin alone, and does not depend upon, or is not interfered with, by the combination of this protein with iron.<sup>1</sup>

*Details of Preparation of Apoferritin*—Two modifications of the method will be described. The first was used for the majority of our preparations. The other was developed only recently. It has the advantage of requiring much less bipyridine, but the yield of apoferritin seems to be somewhat smaller.

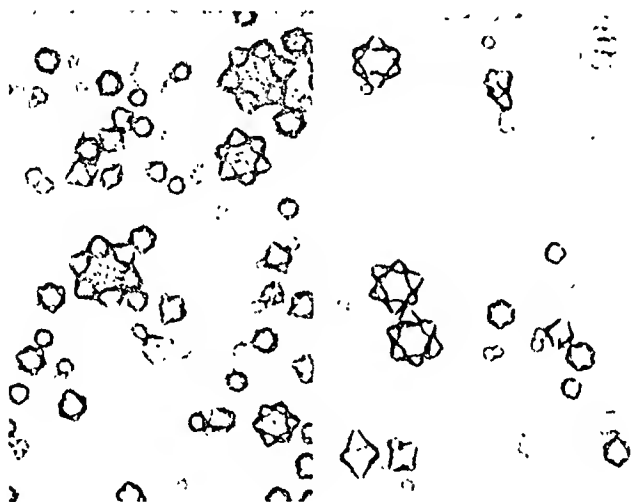


FIG. 1. Left-hand, ferritin (brown); right-hand, apoferritin (colorless);  $\times 250$ .

*Method 1*—15 cc. of a ferritin solution, containing 3 per cent ferritin in dry weight, are placed in a cellophane bag of 1 cm. diameter, together with 100 mg. of powdered  $\alpha, \alpha'$ -bipyridine (which is somewhat more satisfactory than *o*-phenanthroline). The cellophane bag is suspended in a narrow glass tube of 100 cc. capacity containing acetate buffer of pH 4.6 and ionic strength  $\mu = 0.05$ . A current of nitrogen, freed from oxygen, is bubbled through the outer solution. When the oxygen content has decreased sufficiently, 100 mg. of  $\text{Na}_2\text{S}_2\text{O}_4$  are added to the outer solution; the nitrogen is kept bubbling overnight. The iron, in the form of the pink bipyridine

<sup>1</sup> X-ray studies by Dr. I. Fankuchen, which will be published later, show that the arrangement of the protein molecule is identical in crystalline ferritin and crystalline apoferritin.

ferrous complex,<sup>2</sup> diffuses out of the bag. The bag is then dialyzed against distilled water until the diffusate is colorless. Now another portion of bipyridine is added and the whole procedure repeated. By this time the originally dark coffee-brown solution has faded to a pale brown. From this solution, crystals of ferritin with a largely diminished iron content are obtained by adding a solution of  $\text{CdSO}_4$ , enough to make the final concentration of this salt about 5 per cent. These pale yellowish brown crystals are dissolved in a 2 per cent ammonium sulfate solution and again subjected to the whole procedure. Finally, a colorless solution is obtained which, on addition of cadmium sulfate, yields crystals of apoferritin, colorless, iron-free, but in shape indistinguishable from those of brown ferritin. The crystals are not always entirely colorless. A few times they happened to be very pale yellow, which may have been due to a slight contamination with a decomposition product of the bipyridine, formed when the solution was not acid enough. Attempts to remove the colored admixture by recrystallization or by other means were unsuccessful.

*Method 2*—In this method only one treatment with hydrosulfite and one with hydrosulfite plus bipyridine are required.

40 cc. of a 1.6 per cent ferritin solution are placed in a cellophane bag of 1 cm. diameter. This bag is inserted into a long glass tube, 2.5 cm. in diameter and 150 cc. in capacity, filled with acetate buffer (as with Method 1). 1 gm. of sodium dithionite is added with a few glass beads and the tube is stoppered tightly, inclusion of air bubbles being avoided. After 24 hours of intermittent stirring the brown solution becomes pale yellow. (The pH of the outside solution then was 5.23.) The bag is now dialyzed for 24 hours against running distilled water and the contents are treated with cadmium sulfate, whereby pale yellow crystals are yielded. They are dissolved, placed in another cellophane bag containing 300 mg. of  $\alpha, \alpha'$ -bipyridine, and the bag is placed in a tube containing the acetate buffer and sodium dithionite as described for Method 1. The bag is allowed to stand for 24 hours with occasional stirring. It is then removed from the tube and dialyzed and finally the apoferritin crystallized with cadmium sulfate.

*Physical Properties of Apoferritin*—Ultracentrifugation of apoferritin<sup>3</sup>

<sup>2</sup>  $\alpha, \alpha'$ -Bipyridine can be readily regenerated from the Fe-bipyridine complex outside the bag by concentrating the iron complex, making it 2 N with respect to NaOH, and steam-distilling off the bipyridine. The distillate is made acid, evaporated down to a small volume, made alkaline, and extracted with ether. The ether solution is dried with anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated over concentrated  $\text{H}_2\text{SO}_4$  *in vacuo*. The bipyridine is dissolved in a small amount of hot alcohol, filtered, and precipitated by adding water. The product is pure white; m.p. 69.5°.

<sup>3</sup> The ultracentrifugation of apoferritin will be described in detail by Dr. A. Rothen in another paper of this series.

shows that this iron-free substance, in sharp contrast to ferritin, is to the extent of 99 per cent a homogeneous protein with a molecular weight of about 500,000. The remaining heavier protein, amounting to 1 per cent, may be considered as an impurity.

The property of reversible precipitation by heat, such as was described for ferritin, is not shown by apoferritin. However, like ferritin, its solution is irreversibly denatured and coagulated above 80°. Apoferritin is salted-out with ammonium sulfate in the same range as ferritin (*i.e.* between 15 and 30 gm. of the salt per 100 cc. of solution) and crystallizes with cadmium sulfate under the conditions which are also best for crystallizing ferritin.

*Insertion of Iron into Apoferritin*—A successful synthesis of ferritin from apoferritin and a suitable iron compound should be expected to clarify the constitution of ferritin. An essential part of this task is to discover the kinds of iron compounds that can combine with apoferritin to form ferritin. Since in ferritin there is almost 1 iron atom for each peptide group, Kuhn *et al.* have suggested that each peptide group of the protein may be linked with 1 molecule of ferric hydroxide, presumably of the composition  $\text{FeOOH}$ . The ultracentrifuge experiments on ferritin, however, indicated that the iron might be present in the form of colloidal iron hydroxide micelles which are loosely bound to the apoferritin molecules.

Direct interaction of apoferritin with ferric chloride or ferric ammonium sulfate did not result in the formation of ferritin, but only in the coagulation of the protein. Various kinds of colloidal ferric hydroxide solutions which were tested also lacked the property of combining with apoferritin in the desired manner. All of them precipitated in the presence of 5 per cent cadmium sulfate. A detailed description of the various iron solutions used will be omitted here in view of the negative results. They will be characterized in a later paper dealing with various kinds of ferric hydroxide. Suffice it to say that the various colloidal solutions tested differed in color, electric charge, particle size, and magnetic susceptibility. Organic colloidal iron compounds, prepared with yeast nucleic acid, egg albumin, or horse globin, were also tested, all of them without success. The only way that has been found of synthesizing ferritin from apoferritin is as follows:

It has been mentioned (1) that horse spleen always contains more non-hematin iron than corresponds to the ferritin content of the spleen. After ferritin is removed by crystallization with cadmium sulfate, there remains a brown mother liquor which cannot be made to yield further crystals. It contains a brown, colloidal, non-dialyzable iron compound which was designated in Paper I as "non-crystallizable ferritin." When this brown solution is mixed with apoferritin, addition of cadmium sulfate causes the almost immediate formation of typical brown ferritin crystals. It is likely,

therefore, that a specific colloidal iron compound present in "non-crystallizable ferritin" combines with apoferritin to form ferritin crystals. When the brown solution of "non-crystallizable ferritin" is added not to a solution, but to a suspension of apoferritin crystals under a cover slide, the crystals do not stain brown except perhaps at the surfaces. On the other hand, when a dilute solution of crystal violet is added to the suspension of apoferritin crystals, the crystals are rapidly and intensely stained throughout. This indicates that the iron in "non-crystallizable ferritin" is in a colloidal state and is not able to diffuse into the crystal lattice spontaneously.

*Analysis of Apoferritin*—The analytical data on dialyzed apoferritin, dried for 24 hours at 80° and for 3 hours at 110°, are those of atypical protein: N (Kjeldahl) 16.1 per cent, total S (Carius) 0.97, C 51.3, H 7.09, ash 0.91, P (colorimetric, phosphomolybdate) 0.03, Cd (dithizone titration) 0.73, Fe (colorimetric, phenanthroline) less than 0.01.

Because apoferritin is precipitated by cadmium sulfate, it seemed desirable to know whether any of the sulfur is sulfate ion, tenaciously clinging to the protein even after long dialysis. The following experiment was made to clarify this problem. A solution of apoferritin was precipitated with 5 per cent trichloroacetic acid. It may be expected that any sulfate ion clinging to the precipitated protein would be displaced by trichloroacetic ion and the freed sulfate could then be detected in the supernatant liquid. No sulfate was found, however, except for the slight trace contained in the trichloroacetic acid itself.

*Recovery of Apoferritin from Ferritin*—Ferritin Solution IX made from crystalline material and dialyzed and containing a total of 25.56 mg. of N, was treated with bipyridine and sodium dithionite as in Method 1. The two crystallizations with cadmium sulfate were prolonged to 48 hours. The crystals of apoferritin were centrifuged down and dissolved in a 2 per cent  $\text{Na}_2\text{SO}_4$  solution. A slight residue, looking like a denatured protein, was removed by centrifugation. The solution contained 21.5 mg. of N (Kjeldahl). This is a recovery of 84.2 per cent of the nitrogen of the starting material.

In another experiment, from a solution of 320 mg. of ferritin containing 34.2 mg. of nitrogen, 85.4 per cent of the protein nitrogen was recovered as apoferritin by the same method. If one assumes that the nitrogen remaining in the mother liquors of the two crystallizations is also apoferritin nitrogen, the recovery would rise to 92 per cent.

	mg.
Total N in original ferritin solution.....	34.2
" " " apoferritin crystals.....	29.3
" " " Mother Liquor I (from 1st crystallization).....	1.30
" " " " " II (from 2nd recrystallization).....	0.95

By Method 2 in one experiment a recovery of 75 per cent of the nitrogen was obtained.

*Colloidal Iron Compound in "Non-Crystallizable Ferritin"*—An analysis of "non-crystallizable ferritin," i.e. the brown mother liquor remaining after the crystallization of ferritin (see (1), Table III, Preparation VII), reveals that the relative nitrogen, phosphorus, and iron values do not differ appreciably from ferritin itself. It is likely that the iron in that brown solution is present as a colloidal ferric hydroxide, the micelles of which may contain, to some extent, anionic constituents other than OH, and that these micelles are loosely attached to proteinaceous material, including some denatured apoferritin. There is no method available as yet for separating the colloidal ferric hydroxide from its concomitant material, other than to withdraw it by its specific affinity for apoferritin. From the evidence presented in Paper I (1), it is likely that the ferric hydroxide micelle contains all the phosphorus of ferritin, which is in the ratio of 1 P to 8 or 9 Fe. This specific colloidal iron compound is characterized by two distinctive physical properties. One is its magnetic susceptibility, which has a very unusual value for a ferric compound. This will be discussed in a later paper. The other property is its ability to withstand coagulation by salts, especially by 5 per cent cadmium sulfate.

#### DISCUSSION

One of the obvious problems which presents itself is how the iron atoms, which make up 23 per cent of the dry weight of ferritin, are attached to the protein. The facts presented in the preceding pages are scarcely compatible with the hypothesis proposed by Kuhn *et al.* that each iron atom is attached to one peptide group of the protein; that is to say, that the iron is atomically dispersed throughout the protein. The facts against such a hypothesis are these. The splitting of the brown iron-containing protein into a colorless, iron-free protein and an iron-containing residue can be brought about merely by ultracentrifugation. The solution of ferritin does not behave, on ultracentrifugation, as a homogeneous solution. On the other hand, synthesis of ferritin occurs when apoferritin is mixed with "the specific iron solution" which obviously contains the iron not in a molecularly dispersed form but in a colloidal state. The crystal form is independent of whether apoferritin is iron-free or more or less saturated with iron. The facts presented here are compatible with the assumption that in the crystals of apoferritin the interstices are filled with micelles of the specific iron compound. In solution, the micelles adhere to the protein molecule but the binding is so loose that they can be separated merely by centrifugal force. Our hypothesis is also compatible with the inhomogeneity of ferritin demonstrated in the phase rule solubility test, and with the variability of its composition with respect to Fe, N, and P.

The property which renders the colloidal iron hydroxide present in the "non-crystallizable ferritin" alone suitable for the combination with apoferritin cannot be stated as yet. However, no other iron compound was found to be able to combine with apoferritin in the desired manner. Whether this specific property is due to a specific spatial configuration of an iron hydroxide micelle or, in part, to some unknown anionic constituent of the micelle cannot be decided as yet.

That inhomogeneity of a ferritin solution does not show up on electrophoresis is a remarkable fact which still awaits an explanation, but it is not contradictory to the fact that inhomogeneity is shown by other methods.

In conclusion, it may be pointed out that apoferritin is a homogeneous protein which, in spite of its high molecular weight, lends itself to rapid crystallization as a cadmium salt.

#### SUMMARY

Ferritin, a protein of brown color, containing over 20 per cent of iron in the ferric state, and crystallizable as a cadmium salt, can be freed from its iron by reduction to the ferrous state and removal by dialysis after combination with  $\alpha, \alpha'$ -bipyridine. An iron-free, colorless protein solution results from which the protein crystallizes in the presence of cadmium sulfate in the same crystal form as does ferritin. This colorless protein is designated as apoferritin. It is, in contrast to ferritin, a homogeneous protein. Ferritin can be regenerated from it by mixing apoferritin with the brown mother liquor remaining after crystallization of ferritin. This mother liquor contains iron in a colloidal state which has not yet been fully characterized. No other iron compound was found to be able to combine with apoferritin to form ferritin. As regards the structure of ferritin, it is suggested that the iron is not atomically dispersed in ferritin, but that micelles of some iron compound, essentially ferric hydroxide, fill the interstices of the apoferritin structure.

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## INFLUENCE OF ADRENALECTOMY AND OF ADRENOCORTICAL STEROIDS ON LIVER ARGINASE\*

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The recent finding that hypophysectomy causes a striking decrease in the liver arginase in rats and that this process can be reversed by the administration of adrenocorticotrophic hormone (1)<sup>1</sup> indicates an important rôle for the adrenal in the hormonal control of arginase activity. It therefore appears of some importance to investigate which of the crystalline adrenocortical hormones could be held responsible for this action. Qualitative and quantitative differences in the activities of these steroids have already been demonstrated in recent years (2, 3). It has become evident that the presence or absence of an oxygen atom on C<sub>11</sub> and on C<sub>17</sub> is of decisive influence on the type of activity to be expected. For this reason steroids with and without such oxygen atoms were included in this study. This was made possible because of the kind interest of E. C. Kendall in this problem; we were thus able to obtain an adequate supply of steroids with an oxygen atom on C<sub>11</sub>; i.e., Compounds B, A, and E (corticosterone, 11-dehydrocorticosterone, and 11-dehydro-17-hydroxycorticosterone). As a representative of the other class, 11-desoxycorticosterone was available in the form of the acetate (DOCA). However, the low solubility of this substance in aqueous media, when compared with that of corticosterone, might be held to invalidate attempts to compare the effects of these two substances and it therefore appeared desirable to use a more soluble derivative of desoxycorticosterone. This was made possible through the kind conveyal of a small amount of desoxycorticosterone sodium succinate by Dr. C. R. Scholz of Ciba Pharmaceutical Products, Inc.

### *Experimental Conditions and Methods*

Immature female rats were employed. They received hormone treatment for 3 days (in one case 4 days) before autopsy. Normal rats were used when 24 to 26 days old, hypophysectomized rats at the age of 33 to 35

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<sup>1</sup> Fraenkel-Conrat, H., and Evans, H. M., *Am. J. Physiol.*, in press.

days, and adrenalectomized rats at the age of 40 days, both types being operated upon when 26 to 28 days old. The rats were fasted for 7 to 8 hours preceding autopsy (starting at 8 a.m.) except in two experiments in which the rats were fasted 24 hours, starting at 1 p.m.<sup>2</sup> Preceding the fast, food in the form of a wet paste was given *ad libitum*, except in one experiment in which treated and control rats were fed identical amounts of diet by stomach tube. The hormones were administered either in sesame oil subcutaneously,<sup>3</sup> two or three times daily, or orally, *i.e.* both in solution in the drinking water and mixed with the diet. Hypophysectomized rats of the type used drink so amply that administration with the drinking water insured a sufficient supply of hormone, even in the case of the very insoluble DOCA. To administer sufficient amounts of the various compounds orally to normal rats which drink considerably less, the steroids were also introduced into the diet, either by using their aqueous solution for the usual wetting of the diet or by mixing and homogenizing the dry diet in an ether solution of the steroids, then allowing the ether to evaporate, and wetting the diet to a paste as usual. The latter method was used in an experiment in which it was important to insure the administration of identical amounts of hormone to each animal of several groups of rats which were being fed by stomach tube only (Table III, A). While it has been recognized that the steroids with an oxygen atom on C<sub>11</sub> are if anything more active when given orally than parenterally, the effectiveness of DOCA upon oral administration has been questioned on the basis of experiments in which this hormone was given in sesame oil once daily by stomach tube (4). Evidence will be presented elsewhere<sup>4</sup> confirming Grollman's finding (5) that the almost continuous oral administration of the hormone mixed with drinking water and diet leads to the expected hormonal effects. Since in most experiments only the average arginase content of the pooled livers of groups of three rats was determined, it also appeared of importance merely to establish the average amount of hormone consumed daily by each group of rats, thus obviating the need for individual cages. To determine the variability in the arginase content of individual rats within groups, single livers were analyzed in a number of experiments. In those cases equal dosage was insured either by parenteral administration or by giving the hormone mixed with the food by stomach tube. In such experiments it was found that, in groups of four rats, a positive difference (increase) of 33 per cent and a negative one of 25 per

<sup>2</sup> The arginase of untreated rats is 30 per cent higher after 8 hours than after 24 hours fasting (Fraenkel-Conrat and Evans, unpublished experiments).

<sup>3</sup> Sesame oil alone was found to have no effect on liver arginase at the doses used.

<sup>4</sup> Fraenkel-Conrat, H., *Proc. Soc. Exp. Biol. and Med.*, in press.

cent were statistically significant ( $P < 0.05$ ); an increase of 50 per cent was found highly significant ( $P < 0.01$ ).

The rats were anesthetized with sodium amytal before excision of the livers. These were partly used for glycogen determinations,<sup>5</sup> while 0.4 to 1 gm. of each liver was immediately placed on ice for arginase assay. In the rats which had been operated on, complete removal of the pituitary or adrenal was ascertained before the liver samples of a given group were pooled, those from animals in which removal of the organs was doubtful or incomplete being omitted. The liver tissue was broken up into a fine suspension in a Waring blender, with 100-fold of water; arginase determinations on the centrifuged solutions were carried out according to the method of Takehara (6). The arginase activity per gm. of liver was calculated and expressed in units according to Edlbaecher and Rothler (7). Since activators were neither added nor removed from the liver extracts, the determinations give a measure of arginase activity rather than of the concentration of the enzyme in these livers. Whenever in this discussion arginase content or concentration is mentioned, reference is made to the naturally activated arginase or the arginase activity encountered in the tissue. Since the ratio of liver weight to body weight did not vary greatly in similarly fasted groups and since the arginase content of organs other than the liver is comparatively small, this method of expressing activity of liver arginase permits comparative estimates of the total arginase of various types of rats. Actually, in the groups included in this paper, the livers of normal rats, fasted for 8 hours, varied in weight from 3.9 to 4.7 per cent of the body weight, in hypophysectomized rats from 3.4 to 4.1 per cent. The only definite changes in relative liver weight were the increases produced by high doses of Compound E; the livers of these rats averaged 5.3 and 4.7 per cent of the body weights respectively for normal and hypophysectomized rats.

### Results

The experimental results are summarized in Tables I to IV. It is evident that Compound E (17-hydroxy-11-dehydrocorticosterone) caused striking increases in the concentration of liver arginase of hypophysectomized and adrenalectomized rats, with lesser effects in normal rats. The lowest dose (0.3 mg. daily) produced a border line effect but dosages of 0.4 to 0.9 mg. regularly produced increases of 50 to 100 per cent in hypophysectomized rats. In both normal and hypophysectomized rats, the highest doses of Compound E were found slightly less effective in increasing

<sup>5</sup> Fraenkel-Conrat, H., Herring, V. V., Simpson, M. E., and Evans, H. M., unpublished experiments.

liver arginase concentration than lower ones; but at these high levels, liver weights were found increased and calculation of the total liver arginase of

TABLE I

*Effect of Adrenal Steroids on Liver Arginase Content of Hypophysectomized Rats, Fasted for 8 Hours*

Immature females (three per group), 26 to 28 days old at operation, were used; treatment was begun 1 week following operation. The hormones were administered for 3 days.

Arginase is expressed in the units defined by Edlbacher and Rothler (7).

Hormone compound	Daily dose*	Arginase per gm. liver		Carbo-hydrate content per 100 gm. rat weight†	Body weight change	Thymus weight
			Change			
	mg.	units	per cent	mg.	gm.	mg.
E (17-hydroxydehydrocorticosterone)	1.1 O.	1840	+39†	580	-7	81
	0.9 "	2060	+56	416		65
	0.6 "	2540	+93	405	-2	135
	0.6 S.	2190	+66	373	+1	98
	0.3 O.	1660	+26		-6	118
A (dehydrocorticosterone)	0.6 S.	2070	+57	331	-2	119
	0.6 O.	2380	+80	339	-4	150
	0.5 "	2290	+74	346	-4	139
B (corticosterone)	0.4 "	2110	+60	233	-5	177
DOCA (desoxycorticosterone acetate)	3.0 S.	1940	+47	426	+6	146
	1.5 "	1550	+17		+2	
	0.5 "	1080	-18		0	
	0.5 O.	1090	-17	293	+4	167
	0.3 "	1160	-12		-2	168
Desoxycorticosterone sodium succinate	0.9 "	1450	+10	264	-1	147
Controls (11 groups)		1320		231	0	167

\* O. indicates administration with the drinking water, S. subcutaneous injection in sesame oil distributed over two or three daily doses.

† Calculated from determinations of muscle and liver glycogen and blood sugar (to be reported by Fraenkel-Conrat, Herring, Simpson, and Evans), assuming that muscle glycogen and blood sugar are both distributed over 50 per cent of the body weight (8).

‡ Livers in this group weighed 4.7 per cent of body weight, in controls 3.8 per cent. The total liver arginase was 8600 units in the treated and 4400 units in the control group; i.e., an increase of 96 per cent. Liver weights were not determined in the group receiving 0.9 mg. of Compound E. They were found similar to the controls in all other groups.

these animals, rather than of the enzyme concentration, indicated also in these groups what appeared to be maximal effects obtainable within 3 days. Corticosterone and its dehydro derivative (Compounds B and A) were

tested and found effective in producing similar increases at dosages of 0.4 to 0.6 mg. daily in hypophysectomized rats, while in normal rats 0.3 and 0.6 mg. of Compound A were ineffective. When similar dosages of Compounds A and E were given by two different methods, administration with the drinking water was found slightly superior in both cases to subcutaneous injection in sesame oil.

In contradistinction to the effectiveness of the hormones with an oxygen atom on C<sub>11</sub>, the derivatives of desoxycorticosterone caused no significant increases in liver arginase in any type of rat at similar dose levels.<sup>6</sup> At

TABLE II

*Effect of Adrenal Steroids on Liver Arginase Content of Normal Rats, Fasted for 8 Hours*

Immature females (three per group), 24 to 26 days old at onset of treatment. The hormone was administered for 3 days, either mixed with the diet and drinking water (O.) or in sesame oil distributed over 3 daily injections (S.).

Hormone	Daily dose	Arginase per gm. liver		Carbo-hydrate content per 100 gm. rat weight	Body weight change	Thymus weight
			Change			
	mg.	units	per cent	mg.	gm.	mg.
Compound E	1.2 O.	3210	+13*	784	-2	39
	0.3 "	3740	+32		+7	158
" A	0.6 S.	2630	-7		+11	151
	0.3 O.	3070	+8		+12	213
DOCA	3.0 S.	2220	-22		+13	161
Desoxycorticosterone sodium succinate	1.3 O.	2840	0	380	+9	216
Controls (2 groups)		2840		353	+8	203

\* The livers in this group weighed 5.3 per cent of body weight, in the controls 4.4 per cent. The total liver arginase is 17,000 units in the treated group, 12,000 in the control; i.e., an increase of 42 per cent. Liver weights were not affected by the treatment in any other group.

low doses, DOCA actually produced decreases in the liver arginase concentration of hypophysectomized rats, as well as at a higher level in normal rats. While these decreases were not very pronounced, they appeared of possible significance in view of the fact that pituitary growth hormone has been shown to cause decreases in liver arginase.<sup>1</sup> Of the adrenal hormones, DOCA appears to favor increases in body weight, while those

<sup>6</sup> In one experiment a high dose of DOCA (3 mg. daily) caused 47 per cent increase in liver arginase in hypophysectomized rats. It appears significant that in this, as also in regard to the maintenance of carbohydrate stores during fasting (see footnote 5), DOCA proved one-fifth to one-eighth as effective as corticosterone and similar compounds.

compounds with oxygen on  $C_{11}$  have the opposite tendency ((2, 3), also Table I). It seems conceivable that the observed decreases in arginase activity are non-specific and that they might naturally accompany any

TABLE III

*Effect of Adrenal Steroids on Liver Arginase Content of Hypophysectomized and Adrenalectomized Rats, Fasted for 24 Hours*

Immature females, 26 to 28 days old at operation. Hormone treatment (A) for 4 days, starting 6 days after hypophysectomy; (B) for 3 days, starting 14 days after adrenalectomy. All rats in (B) received 1 per cent NaCl solution to drink during the preexperimental period, and in all but one group during the period of hormone treatment. O. oral; S. subcutaneous.

Hormone*	Daily dose	Arginase per gm. liver		Carbohydrate content per 100 gm. rat weight	Body weight change	Thymus weight
			Change			
(A) Hypophysectomized rats on identical food intake†						
	mg.	units	per cent	mg.	gm.	mg.
Compound E (4)	0.66 O.	1660	+79	227	-4	56
DOCA	0.65 "	1000	+8	208	-3	173
Controls (4)‡		930		236	-1	115
Normal controls (5)		1860	+100	338	-4	150
(B) Adrenalectomized rats, maintained on 1% NaCl						
Compound E (5)	1.0 S.	1090	+85	289	+11	329
DOCA (4)	1.0 "	730	+24	268	+15	368
Controls (4)	Salt, as above	590		211	+5	389
" (2)	Without salt for 4 days	580		221	+2	348
Normal controls (5)	On salt	1900	+220	279	+6	365

\* The figures in parentheses indicate the number of animals per group. Individual livers were analyzed in this experiment. The individual arginase concentrations were 1430, 1470, 1830, 1910 units (Compound E); 740, 980, 1100, 1180 units (DOCA); 670, 800, 1060, 1180 units (hypophysectomized controls).

† The hormone was mixed with the diet (only 3 gm. per day) and administered by stomach tube approximately every 8 hours; only 0.4 and 0.15 mg. of Compound E and DOCA, respectively, were given during the fasting period, in aqueous solution by stomach tube.

‡ The average arginase content of eighteen groups of livers of similar rats fed *ad libitum* preceding the 24 hour fast is 1000 units per gm.

positive nitrogen balance with its concomitant disuse of the urea-forming enzyme systems. Conversely, the increases in arginase provoked by Compounds A, B, and E might well be secondary to an increased rate of glyconeogenesis and protein catabolism, leading to a negative nitrogen bal-

ance. The question poses itself, do hormonally produced changes in arginase activity represent homeostatic adjustments to changes in nitrogen metabolism or are they primary in nature, leading secondarily to the changes in the nitrogen metabolism? Two findings favor the latter interpretation. Thus pronounced increases in liver arginase were obtained even with levels of the steroids inadequate to stimulate sufficient glycogenesis for the maintenance of carbohydrate stores during fasting (Table I, corticosterone; Table III, A, Compound E). Further, arginase was also found to be increased by Compound A in two groups of rats which were not fasted and were in nitrogen balance.<sup>7</sup> Thus it would appear

TABLE IV

*Effect of Gonadal Steroids on Liver Arginase Content of Hypophysectomized Rats, Fasted for 8 Hours*

The rats were of the same type as in Table I. The hormones were given subcutaneously in sesame oil.

Hormone	Daily dose	Arginase per gm. liver	
			Change
	mg.	units	per cent
Estradiol dipropionate	0.5	980	-26
" "	0.01	1360	+3
" benzoate	0.5	1300	-2
Progesterone	0.5	1520	+15
Testosterone propionate	0.5	1670	+27
" "	0.5	1490	+13
" "	1.0	1490	+13
Controls (from Table I)		1320	

probable, though by no means proved, that corticosterone-like compounds directly effected changes in arginase activity which in turn produced or contributed to an increased rate of protein catabolism and glycogenesis.<sup>8</sup>

In view of the influence of diet and food intake on liver arginase concentration (10), it appeared important also to study the effect of adrenal steroids in rats receiving identical amounts of food. Thus hypophysectomized and normal control rats were fed only by stomach tube for 4 days

<sup>7</sup> Hypophysectomized rats received 0.3 mg. of Compound A orally for 3 days. The treated animals consumed more food and excreted correspondingly more nitrogen. Their carbohydrate stores were found increased at autopsy (450 mg. per 100 gm. of rat, compared to 350 mg. in the controls); body weights remained unchanged in all rats. Liver arginase of the treated rats was 40 per cent higher than in the controls.

<sup>8</sup> Evidence for a similar action of adrenal steroids on amino acid oxidase has been reported (9).



preceding a 24 hour fast (Table III, A). Also under these conditions, Compound E produced a great increase in the liver arginase of the hypophysectomized animals, causing it to approach the level in normal rats, while DOCA showed no significant effect.

A number of gonadal steroid hormones have been included in this study for purposes of comparison. None of these was found to have consistent or pronounced effects when administered at levels comparable with those employed with the adrenal steroids (Table IV). After these experiments had been completed, an article appeared reporting increases in arginase activity following prolonged administration of testosterone propionate to castrated rats (11). An insignificant tendency in this direction appears also in our experiments which differ in many respects from those quoted above. On the basis of the demonstration by Edlbacher *et al.* (12) of arginase activity in tumor tissue, Kochakian and Clark (11) attempted to correlate the observation of an increase in arginase activity with the previously demonstrated nitrogen retention and growth effects of testosterone. This appears untenable in view of all our recent findings concerning hormonal control of liver arginase in a standardized test object; growth was always found to be accompanied by decreases, protein catabolism by increases in this enzyme system.<sup>9</sup> It appears probable that the increase of liver arginase from high doses of testosterone may be due to its chemical resemblance to the adrenal steroids or be mediated by another endocrine gland.

The striking ability of certain adrenal hormones to increase the liver arginase concentration of rats indicated the importance of studying the effect of adrenalectomy on this enzyme system. Liver arginase was therefore determined in immature adrenalectomized rats, 17 days after the operation (Table III, B). All rats received 1 per cent sodium chloride to drink for 2 weeks after the operation and, as is usual, continued to grow under this treatment. The adrenalectomized rats were then divided into four groups, three of which were continued on salt, while one received only tap water and a salt-free diet. Two of the salt-treated groups received 1 mg. daily of Compound E and DOCA, respectively. A control group, not operated on, was given salt water to drink. All rats were fasted for 24 hours preceding autopsy. Analyses of their livers indicated a pronounced decrease in activity of liver arginase following adrenalectomy,

<sup>9</sup> While arginase has been demonstrated to be associated with rapidly growing tissues (12), it seems to occur there only in small amounts, as compared with its concentration in mammalian livers. Its function also appears to be entirely different in tumor and in liver tissue. It has never been claimed by Edlbacher and his associates that increased liver arginase accompanies general growth processes, but only that this enzyme can be detected in tissues of high mitotic activity.

lowering it to one-third that of the normal rats; these levels are even below those observed following hypophysectomy. Salt treatment alone did not effect a change, while a slight rise was produced by DOCA and a pronounced increase by Compound E.

#### DISCUSSION

A considerable body of knowledge concerning the differential activities of various adrenal steroids has accumulated in the last few years and has been summarized in recent reviews (2, 3). The presence of an oxygen atom on  $C_{11}$  was found greatly to increase the capacity of these steroids to cause the changes in carbohydrate and protein metabolism characteristic of the adrenal cortex. Increased rate of glycconeogenesis and protein catabolism, contrainsular, "diabetogenic," glycostatic, and glycotropic activities have all been described. These and several other effects which are not so clearly connected with this group of phenomena, such as increased work performance in the Ingle test, thymus atrophy, etc., are produced much less effectively if at all by desoxycorticosterone and the amorphous fraction.

To the series of related carbohydrate and protein metabolic effects of the adrenal, another has now been added in the demonstration of the dependence of liver arginase activity upon the adrenal. It has also been shown that an oxygen atom on  $C_{11}$  is favorable to, if not essential for, the action of adrenal steroids in bringing the liver arginase of hypophysectomized or adrenalectomized rats back to normal or increasing it to supernormal levels. On the other hand the presence or absence of an oxygen atom on  $C_{17}$  did not appear to affect this activity. The amount of hormone needed for the arginase effect has been found to be similar to or possibly slightly lower than that necessary to produce the other changes. There are indications that the arginase-increasing action (probably in conjunction with effects on other enzyme systems) is primary and responsible for many of the metabolic effects of the adrenal cortex which have been previously recognized and described.

Attention may be drawn to the similarity in the effects of cortical steroids and of the pituitary adrenocorticotrophic factor in the control of liver arginase. Here, as in all other aspects, the pituitary principle resembles in its action those adrenal steroids which have an oxygen atom on  $C_{11}$ , rather than desoxycorticosterone. Swann (13) in his interesting review of the pituitary-adrenocortical relationship has classified the functions of the adrenal cortex as slightly, partially, or greatly influenced by the pituitary gland. From knowledge which has since accumulated, it appears as if all adrenal functions which are greatly influenced by the pituitary can be reproduced by administration of the steroids with an oxygen atom on  $C_{11}$ ,

and less effectively if at all by other hormonal components of adrenal extracts. It would thus appear that the hypophysis controls primarily the secretion of a hormone or hormones structurally related to corticosterone; *i.e.*, containing an oxygen atom on C<sub>11</sub>.

#### SUMMARY

1. Adrenalectomy was shown to cause a marked decrease in the arginase activity of rat livers, exceeding that which has been observed following hypophysectomy.

2. Administration of small amounts of corticosterone, 11-dehydrocorticosterone, and 11-dehydro-17-hydroxycorticosterone produced increases of the liver arginase in normal, hypophysectomized, and adrenalectomized rats.

3. Deoxycorticosterone, administered at similar dose levels, was shown to have no or a slight arginase-decreasing action in all three types of rats.

4. No significant effects were produced by male and female sex hormones at similar dosages.

5. Attention was directed to the probability that the effect of the adrenocortical hormones on certain enzyme systems, such as that here studied, is basically responsible for the rôle of this gland in the control of carbohydrate and protein metabolism.

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# A NEW METHOD FOR THE MICRODETERMINATION OF IODINE IN CERTAIN BIOLOGICAL MATERIALS

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In the course of studies on antigen-antibody reactions, with iodoproteins as the antigen, it was realized that a quick, accurate method for the determination of iodine would be of great value. With this purpose in mind a number of powerful oxidizing agents were studied, and it was found that potassium chlorate in sulfuric acid would give excellent results. After studies with regard to the most suitable quantities and optimum ratios of these two reagents, a standard method was developed which is described in this paper. The procedure should not be considered as absolutely rigid. The quantities of sulfuric acid and potassium chlorate as well as their ratio can be changed to suit the purpose.

The method is simple to operate, and does not require any special equipment. Many samples can be run at the same time and determination can be completed in a short period of time. Small quantities of reagents are used which can easily be freed from iodine,<sup>1</sup> thus minimizing the danger of iodine contamination. Digestion is carried out at comparatively low temperatures, so that no loss of iodine occurs due to vaporization. It has the accuracy of 0.8  $\gamma$  for one determination, 0.5  $\gamma$  for the average of two determinations, and 0.2  $\gamma$  for the average of three determinations. Any percentage of iodine in the compound can be determined, as long as the dry weight of the sample does not exceed 30 mg. This method is not readily adapted to the analysis of blood or tissues which contain certain metals, e.g. iron or manganese, but it will probably be a reliable and useful procedure under appropriate circumstances for many routine analyses upon a well known type of material. Although the writer has successfully used this method for iodoproteins containing as high as 13.1 per cent iodine, the method is not as convenient as the Groök permanganate oxidation for iodoproteins containing over 5 per cent of iodine. Its chief advantage lies in the analysis of material containing from 0.05 to 1 per cent of iodine; e.g., for analysis of thyroid preparations. Even so, it is not so accurate as the Kendall-Hunter method, which, however, requires an hour's time and much more material.

<sup>1</sup> Sulfuric acid can be made iodine-free if concentrated sulfuric acid is boiled for 10 minutes. Potassium chlorate can be purified by dissolving it in boiling water, and then allowing it to cool and crystallize. Crystallization is carried out three times.

In this method the material to be analyzed is digested with potassium chlorate in sulfuric acid,<sup>2</sup> which at the same time oxidizes the iodine in the compound to iodic acid. Soon after heating, chlorine oxides are produced which decompose at higher temperatures, leaving a clear colorless liquid. After dilution the traces of chlorine are removed from the solution, and the iodine is released by the addition of potassium iodide solution and determined by titration with sodium thiosulfate.

### *Method*

One to twelve samples can be run at a time. The digestion is carried out in micro-Kjeldahl digestion flasks. As chlorine will be evolved, care should be taken to prevent it from escaping. If inverted rubber stoppers are placed around the necks of the flasks, which are then fitted tightly into the tube of the digestion rack, no difficulty is encountered. The vacuum in the tube of the digestion rack should not be more than a few cm. of mercury; otherwise frothing and bumping may occur.

2 ml. of sulfuric acid (concentrated, sp. gr. 1.84, diluted with an equal volume of distilled water) are added to each of the digestion flasks, followed by 1 ml. of the liquid to be analyzed.<sup>3</sup> If the sample is in solid form, 1 ml. of distilled water should be added with the sample. Owing to the fact that the sample should be in contact with the reagents during digestion, care should be taken that no large particles of solid matter are left on the sides of the flask. The quantity of potassium chlorate to be added depends on the solid content of the sample. For each 10 mg. of dry matter 0.25 gm. of potassium chlorate<sup>4</sup> is added, the limit being 0.75 gm.<sup>5</sup>

Heat the flasks very gently until the liquid becomes green. Continue heating for about 2 minutes (for 0.500 gm. of potassium chlorate heat for 5 minutes, for 0.750 gm. heat for 10 minutes), after which the flame can gradually be increased. If there is any bumping, the flame should be lowered. Boil the liquid until the color fades away and white fumes start

<sup>2</sup> If potassium chlorate crystals are brought in contact with concentrated sulfuric acid, a mixture of chlorine oxides and their acids is produced which is highly explosive; if, however, the sulfuric acid is diluted 2 or 3 times, as long as the potassium chlorate content does not exceed the limit of 0.750 gm., the mixture can be handled with safety.

<sup>3</sup> If there is a possibility of inorganic iodide being present, in order to avoid loss of inorganic iodine it is better to add the reagents without the sample. Heat until the green color is produced, cool for 1 minute, add the sample, and proceed with digestion.

<sup>4</sup> If the potassium chlorate is iodine-free, this amount can be measured by volume of the solid instead of the weight.

<sup>5</sup> For higher solid content as much as 1 gm. of potassium chlorate can be added without any danger if an extra ml. of water is added to the mixture and the heating is carried out with great caution.

to evolve. Continue heating for 1 more minute at the same rate. If the green color returns, continue heating until the liquid becomes colorless or white. Turn off the flame and leave the flasks to cool. The process of digestion takes about 10 minutes for 0.250 gm., 20 minutes for 0.500 gm., and 30 minutes for 0.750 gm. of the added potassium chlorate.

As soon as the flasks are cool enough to permit the addition of water without boiling, 10 ml. of distilled water are added to each flask and the contents then transferred to 50 ml. Erlenmeyer flasks which have been previously marked to the 15 ml. level with a diamond pencil. Wash the digestion flasks twice, each time with 5 ml. of distilled water, and transfer to the corresponding Erlenmeyer flasks. Add a piece of pumice stone to each flask, and boil until the level of the liquids reaches the 15 ml. mark. The digestion rack can be used for this purpose. Cool the flasks in shallow running water and add a crystal of phenol to each flask. Add 1 ml. of 0.5 per cent solution of potassium iodide and titrate immediately, with a solution of freshly prepared 0.001 N solution of sodium thiosulfate. For values of more than 0.2 mg. of iodine use 0.01 N solution of sodium thiosulfate. 1 drop of 1 per cent starch solution is used as indicator. The use of the micro burette will give slightly more accurate results if the titration is completed in a short period of time. As will be explained in the next paragraph, the period of titration is of importance in the accuracy of results. For this reason an ordinary 5 ml. burette will, to some extent, make up for the accuracy of the micro burette because of the speed in titration.

Small amounts of iodine are liberated from the potassium iodide which has been added, owing to the high concentration of acid and the probable presence of some oxidizing agents. Although the rate of release of iodine is very slow, about 0.7 to 1.0  $\gamma$  in 10 minutes, it is important to time all the titrations and finish them at a reasonably constant period of time. As this method is designed to save time, this complication cannot be avoided unless special methods are used, such as liberating the iodine with reducing agents and distilling it over, in which case other methods are preferable. Salter (1), McClendon (2), and Harvey (3) give good reviews and references to many methods for microdetermination of iodine. Also, because of the large quantities of salts and acids present, the starch does not give a very exact end-point. A more accurate end-point can be obtained, however, if after digestion the flasks are allowed to cool to room temperature, and the salts are precipitated with 5 ml. of 85 per cent ethyl alcohol. The precipitate is centrifuged and washed twice with 5 ml. of 85 per cent ethyl alcohol. 5 ml. of distilled water are added to the supernatant fluid which contains the iodate, and the procedure of the driving off of chlorine is followed, only this time care must be taken to

TABLE I

*Determination of Correction Value for Iodine Determination with Iodoacetic Acid*

Digestion was carried out with 0.25 gm. of potassium chlorate. The iodine determination was made on 1 ml. of solution in each case.

(a) 10.6 mg. iodoacetic acid dissolved in 100 ml. distilled water				(c) 25.00 ml. solution (a) diluted to 100 ml.			
0.001 N sodium thiosulfate used	Iodine equivalent	Calculated value	Difference	0.001 N sodium thiosulfate used	Iodine equivalent	Calculated value	Difference
ml.	mg.	mg.	mg.	ml.	mg.	mg.	mg.
3.46	0.0730			0.90	0.0189		
3.43	0.0724			0.86	0.0181		
3.46	0.0728			0.86	0.0181		
Average...	0.0728	0.0724	+0.0004		0.0184	0.0181	+0.0003
(b) 50.00 ml. solution (a) diluted to 100 ml.				(d) 25.00 ml. solution (c) diluted to 100 ml.			
1.72	0.0362			0.20	0.0042		
1.74	0.0367			0.24	0.0050		
1.72	0.0362			0.24	0.0050		
Average....	0.0364	0.0362	+0.0002		0.0047	0.0045	+0.0002
Correction value.....							0.0003

TABLE II

*Determination of Iodine in Thyroid Tablets*

Small pieces of a thyroid tablet (Sharp and Dohme, No. 925) were weighed and the iodine content determined.

Weight	Iodine	Iodine after correction	Per cent of iodine	Difference
mg.	mg.	mg.		mg.
22.3	0.0186	0.0184	0.082	+0.0002
16.4	0.0133	0.0131	0.079	-0.0003
12.7	0.0114	0.0112	0.088	+0.0005
4.7	0.0040	0.0037	0.079	-0.0003
Average.....			0.082	

TABLE III

*Determination of Iodine in Diluted Horse Iodoalbumin*

Dilution	Iodine, average of 2 determinations	Iodine after correction	<u>Diluted</u> <u>Undiluted</u>	Difference
	mg.	mg.		mg.
	0.1232	0.1229	1	+0.0004
2/7 = 0.286	0.0352	0.0349	0.285	0
4/49 = 0.0816	0.0099	0.0096	0.0781	-0.0004

prevent the alcohol vapor from igniting. This procedure can also be used when oxidizing agents which may interfere with the iodide-iodate reaction are present. Small quantities of the salts will, however, be left in the solution.

Owing to the slightly high values obtained in this method, a series of controls should be run. The figure obtained can be used for correction as long as the sulfuric acid and potassium chlorate stock bottles last. Although blanks can be run as controls, it has been found that more accurate results are obtained if iodine determination is made on known quantities of either iodoacetic acid or iodopropionic acid and the average differences between the experimental results and the calculated figures are used for the correction. Table I gives a satisfactory method for the finding of the correction figure. Tables II and III give examples of the range of accuracy of this method. The writer has run nearly a thousand duplicates on iodoproteins and they all fall within the same range of accuracy. Controls should also be made and correction figures obtained for different quantities of potassium chlorate.

The time taken for twelve determinations is  $1\frac{1}{4}$  to  $1\frac{3}{4}$  hours, depending on the amount of potassium chlorate used. When a great number of determinations has to be made, more time can be saved if, while one batch is boiling in Erlenmeyer flasks, another batch is being prepared in digestion flasks, and while the titrations are made for the first batch, the second batch is being digested. In this way the time can be reduced to about 2 to  $2\frac{1}{2}$  hours for twenty-four determinations, giving an average of about 5 to 7 minutes for each determination.

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# THE EFFECT OF PROGRESSIVE IODINATION FOLLOWED BY INCUBATION AT HIGH TEMPERATURE ON THE THYROIDAL ACTIVITY OF IODINATED PROTEINS\*

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The reports of Ludwig and von Mutzenbecher (1) and Harington and Rivers (2) established the fact that thyroxine can be isolated from hydrolysates of iodinated proteins prepared under rather closely defined conditions. Little attention was given, however, to the thyroidal effects to be obtained without preliminary hydrolysis of the iodinated proteins used.

Thyroidal responses indicating a relatively low order of potency were reported for degradation products of iodinated serum albumin by Salter and Lerman (3). Similar effects were obtained by oral administration of iodinated serum albumin itself by Lerman and Salter (4).

In our early experiments it was found that iodinated casein prepared in a medium buffered with sodium bicarbonate exerted thyroidal effects when given either orally or by injection. It was demonstrated (5) that, other conditions being held constant, the thyroidal activity of iodinated casein increases progressively with increasing additions of iodine until sufficient iodine has been added to combine 2 atoms per molecule of tyrosine in the protein. Iodination beyond this point results in a significant loss of thyroidal activity.

In extensive experiments that are being published elsewhere (6), it was found that, if the iodine input was held constant, and the bicarbonate concentration of the reaction mixture was varied, the attainment of maximal thyroidal activity was dependent upon adding sufficient bicarbonate to hold the pH within the range of approximately 6.8 to 8.0. Addition of insufficient bicarbonate resulted in products with low activity; the use of an excess of bicarbonate appeared to have no effect on the result, however.

All of the reactions up to this point had been conducted at the usual temperature of 38°. When the concentrations of both the iodine and the sodium bicarbonate were held within the optimal ranges thus established, it was found (6) that about a 4-fold increase in thyroidal activity could be obtained by incubating the preparations at an elevated temperature of 60-70°. Furthermore, this increase would take place whether the protein was first

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iodinated, and then incubated at the elevated temperature, or the reaction was conducted at the elevated temperature throughout.

The question then arose whether or not the extent of iodination used would limit the reaction under these new conditions in a manner similar to that observed previously. The experiments described in the present report were designed to throw additional light on this question.

#### EXPERIMENTAL

The preparations of iodinated protein were made up exactly as described previously (5) except that the desired amount of iodine was first combined with the protein at a temperature of 38–40° and the temperature was then increased to 70° during the subsequent incubation period of 18 to 20 hours.

Series I was prepared from casein containing 5.65 per cent tyrosine, as determined by the method of Lugg (7). Six different preparations were made up with the amount of iodine added, increasing progressively from 7.5 to 38 gm. per 100 gm. of protein. A soy bean protein kindly supplied by the United States Regional Soybean Industrial Products Laboratory, Urbana, was used in preparing Series II. The tyrosine content of this protein was found to be 4.22 per cent. Graded amounts of iodine ranging from 6.0 to 36.0 gm. per 100 gm. of protein were added in preparing this series.

The thyroidal activity of the iodinated proteins of both series was estimated by injection into frog tadpoles, as described in greater detail elsewhere (6). Each preparation was tested on ten tadpoles, exactly 40  $\gamma$  of iodoprotein from each preparation being injected into the animals of its respective test group. Therefore, the per cent decrease in body length provides an index of the relative potencies of the various members of the series. Three additional groups of tadpoles were injected with a synthetic monosodium salt of thyroxine on graded dosage levels, in order to provide a reference standard to compute roughly the actual thyroidal potency of the experimental preparations. In order to avoid the effect of varying environmental conditions on the tadpole responses, all the animals were injected on the same afternoon.

#### Results

Data on the iodination procedure, iodine analyses, and biological assay are given in Table I. The results obtained with the new incubation temperature of 70° run parallel in all respects to the previous observations (5) on preparations that were iodinated and incubated at 38°.

Here again, approximately one-half of the iodine is combined with the protein until sufficient has been added to substitute 2 atoms per molecule of tyrosine. This agrees with the assumption that the principal reaction during this stage is the substitution of iodine on tyrosine according to the equation,  $\text{tyrosine} + 2\text{I}_2 \rightarrow \text{diiodotyrosine} + 2\text{HI}$ . Furthermore, the

Millon reaction becomes negative at this point, giving further evidence that tyrosine is the principal constituent of the protein involved. After the tyrosine has been fully substituted, iodine is taken up much less readily, with the result that progressively smaller increments of iodine are taken up as more is added.

The biological assay shows that the thyroidal activity starts out at a relatively low level in both series, gradually increases to the maximum when sufficient iodine has been added for substitution of 2 atoms per mole of tyrosine, and then decreases markedly with excessive iodination.

TABLE I  
*Effect of Progressive Iodination and High Temperature Incubation on Thyroidal Activity of Iodinated Proteins*

Series No.	Preparation No.	Iodine added per 100 gm. protein	Iodine added per mole tyrosine	Iodine combined	Iodine combined per mole tyrosine	Tadpole responses	Per cent of thyroxine response	P*
		gm.	atoms	per cent	atoms	per cent		
I. Iodinated casein	1	7.5	1.89	4.11	1.08	16.2	4.17	
	2	12.5	3.16	5.93	1.59	27.2	5.82	<1
	3	19.0	4.80	7.55	2.06	34.9	8.50	<5
	4	25.0	6.31	8.19	2.25	21.6	5.07	<1
	5	32.5	8.21	8.60	2.38	11.8	3.42	1
	6	38.0	9.60	9.13	2.54	4.6	2.40	<5
II. Iodinated soy bean protein	1	6.0	2.03	3.21	1.12	14.2	3.82	
	2	11.5	3.88	5.20	1.85	21.5	5.07	<5
	3	17.5	6.35	6.15	2.22	22.9	5.25	>5
	4	23.5	8.95	6.51	2.35	7.7	2.92	<1
	5	30.0	10.14	7.40	2.70	2.9	2.35	1
	6	36.0	12.17	7.78	2.85	1.6	2.32	>5

\* P = the per cent probability that the difference from the preceding member of the series is due to chance variation.

The significance of the difference between the tadpole responses in successive members of both series was computed as described by Snedecor (8). The probabilities are given in the last column of Table I. In Series I there is less than a 5 per cent chance in all cases that the differences in thyroidal response to successive preparations in both the ascending and descending portions of the activity scale could be due to chance variations in the assay. In Series II, Preparations 2 and 3 were not found to be significantly different, probably because the degree of iodination was too low for maximal activity in Preparation 2 and too high in Preparation 3. However, both of these preparations showed significantly higher activity than the preparations that were iodinated to either a greater or less extent.

In both series there is far higher thyroidal activity at the optimal level of iodination and a much more pronounced decline in activity as iodine is added

in excess of this point than was observed earlier in products made up at the temperature of 38° throughout.

As a quantitative standard for estimating the total thyroidal effect of the iodinated protein preparations, monosodium thyroxine was dissolved with the aid of a minimal amount of NaOH solution and injected into three groups of ten tadpoles at the levels of 1, 2, and 4  $\gamma$  respectively, per animal. The average responses in ascending order of dosage were 2.7, 24.0, and 35.4 per cent. The response to a given preparation of iodinated protein was then computed as the percentage of the response to thyroxine, as described by Reinecke and Turner (6). The results are included in Table I. In Series I the thyroidal activity increases with increasing iodination from 4.17 to 8.5 per cent of that of thyroxine and then declines to 2.4 per cent. A similar trend is shown by the iodinated soy bean protein of Series II, although at a somewhat lower level of potency. The bulk of the evidence accumulated to date indicates that the formation of thyroxine from non-descript proteins depends upon the iodination of tyrosine, followed by its conversion into thyroxine. Inasmuch as the tyrosine content of the casein used in Series I was 5.65 per cent as compared to 4.22 per cent tyrosine in the soy bean protein, it would be expected that the casein preparations would have higher activity at a given level of iodination.

#### DISCUSSION

The present report fully confirms our previous finding that the formation of iodinated proteins with maximal thyroidal activity is dependent upon limitation of the iodine input to an amount sufficient only for substitution of 2 atoms per molecule of tyrosine. This is true when the process is carried out entirely at 38° as well as when the iodination mixture is incubated at 70°.

The preparations treated with optimal iodine concentrations and incubated at high temperature have uniformly shown surprisingly high thyroidal activity when assayed by injection into tadpoles. Preparation 3, Series I, was found to have an apparent activity of 8.5 per cent that of thyroxine. In other experiments (6) we have repeatedly observed responses of the same order with products prepared under similar conditions. In fact a number of preparations made up in the presence of metals that apparently catalyze the reaction (6) have exerted thyroidal effects equivalent to about 11 per cent of the response to thyroxine. That these responses are not due to a special reaction of tadpoles is shown by the fact that similar responses have been obtained in our assays of selected preparations in guinea pigs by both oral and parenteral administration. When compared with thyroxine given by the same routes, iodinated proteins have shown 2.7 times as much activity when injected as when given orally.

It now becomes of interest to inquire into the nature of the substance exerting such a pronounced thyroidal effect in these preparations. Thyrox-

ine can be isolated from them readily, along the lines described by Ludwig and von Mutzenbecher, although it is doubtful that the amount of this substance present will account for the thyroidal effects obtained. In unpublished experiments we have been able to isolate as much as 424 mg. of crystalline thyroxine of good purity from 100 gm. of iodinated casein. In spite of the sizable losses involved in the isolation of thyroxine, much higher yields should be obtained if all the activity shown by the biological assays were due to this substance. In view of the higher activity of these iodinated proteins when injected than when given orally, it appears likely that the thyroxine formed as a result of iodination remains combined within the protein molecule in an active state. If this state of combination were to contribute to its activity, as suggested for thyroid hormone by Harington (9), it would be possible to obtain iodoproteins with higher thyroidal activity than would be accounted for by the highest possible amounts of thyroxine that could be isolated from them.

#### SUMMARY

Two separate series of preparations, one with casein, and the other with soy bean protein as substrate, were iodinated progressively, followed by incubation at 70° for 18 to 20 hours. All preparations were assayed by injection into frog tadpoles. In confirmation of previous work, carried out at a lower temperature, the thyroidal activity of both series increased with increasing iodination until sufficient iodine had been added to substitute 2 atoms per molecule of tyrosine in the protein. Iodination beyond this point resulted in pronounced decreases in thyroidal activity. Casein that was iodinated to the optimal level, followed by incubation at high temperature, produced an assay response of 8.5 per cent of that of thyroxine. Soy bean protein treated similarly showed a maximum of 5.25 per cent of the activity of thyroxine. The high thyroidal activity of these preparations is discussed in relation to the yields of thyroxine to be obtained from them and the methods of assay used.

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# THE EXTRARENAL REGULATION OF MUSCLE AND SERUM POTASSIUM FOLLOWING EXTRACELLULAR FLUID AND SODIUM DEPLETION\*

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During an investigation of shifts of water from the extracellular to the intracellular space in sodium-depleted rats it was observed that the concentration of potassium in the serum and skeletal muscle was increased within 4 or 5 hours of the onset of the depletion. Yannet and Darrow found similar increases in the potassium of the serum and muscle 24 to 48 hours after severe depletion of sodium (1). They attributed their findings to an impaired excretion of potassium by the kidneys, since oliguria was marked. In the present study the shorter duration of the experiments and the occasional presence of a considerable diuresis made it possible to exclude the renal factor in the interpretation of the results and made it necessary to seek some other explanation for the rise in serum and muscle potassium. Subsequent experiments revealed that the types of injury employed in this study altered the metabolism of the muscle cell in such a way as to produce an increase in muscle potassium concentration. The types of injury used in the present study were mild and primarily involved either a partial depletion or an immobilization of a portion of the animal's extracellular electrolyte. In their severe forms the same injuries are associated with hemoconcentration and a reduction of blood volume and pressure. In addition to establishing a new concept of potassium regulation the present experiments offer a satisfactory explanation for the rise in serum potassium observed clinically following burns and other shock-producing injuries.

## Methods

Male albino rats weighing from 250 to 350 gm. were used throughout the experiments. They were maintained on Purina dog chow and tap water. Rats were removed from their cages and injected intraperitoneally with a 5 per cent solution of glucose or gum acacia<sup>1</sup> in an amount equal to 5 cc. per 100 gm. of body weight. 4 to 5 hours after the injection the rats were sacrificed. In all instances in which glycogen determinations were

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<sup>1</sup> The acacia was found by analysis to be free of sodium.



made an intraperitoneal injection of nembutal in amounts up to 50 mg. per kilo of body weight was used to anesthetize the animals. Otherwise the rats were sacrificed under ether anesthesia. Rats were also removed from their cages and scalded while under ether anesthesia by placing them in a water bath at 90° from 4 to 6 seconds, usually 5 seconds, so that the skin over the back and flanks was included in the scalded area, but not the head, extremities, or abdomen. The scalded animals were sacrificed from 1 to 16 hours later, usually 4 to 5 hours. A moderate second degree burn was produced in all animals. Because of the possible importance of a decrease in muscle glycogen in relation to the increase in muscle potassium a group of rats was removed from the cages and injected subcutaneously with 0.05 mg. of adrenalin chloride solution (Parke, Davis) and sacrificed 3 to 4 hours later (2). Rats were also fasted 24 hours and then scalded or injected with 0.05 mg. of adrenalin chloride solution. One group of rats fasted for 24 hours served as controls. All fasted rats were sacrificed, intraperitoneal nembutal being used for anesthesia.

Glycogen determinations were made on the gastrocnemius muscle and a slice of the liver according to the method of Good, Kramer, and Somogyi (3). After muscle and liver had been removed for glycogen studies, blood was withdrawn under oil in a syringe from the abdominal aorta and the serum was separated from the cells soon after the blood clotted. The serum was analyzed for water, sodium, chloride, and potassium. Muscle was cut from the hind legs for the determination of water, fat, nitrogen, sodium, chloride, potassium, and phosphorus. Occasionally the liver was also analyzed for electrolytes. Urine collections were made on some of the rats injected with glucose and acacia solutions and the urine was analyzed for chloride, sodium, potassium, and nitrogen. None of the scalded rats voided. The peritoneal fluid removed from rats receiving glucose and acacia was also analyzed for electrolyte in some instances. The methods used for electrolyte determinations were the same as those previously used in this laboratory (4).

### *Results*

The results of the experiments on the fed rats are summarized in Table I. In the fed rats in Groups II to V the concentrations of muscle potassium and phosphorus are significantly increased over those of the control group. The muscle nitrogen also is significantly increased in the glucose- and acacia-injected animals and is elevated in the scalded rats but not to a statistically significant extent. There is definite evidence of sodium depletion in the glucose-injected rats of Group II, since the concentrations of sodium and chloride in the serum are significantly lower than normal. The loss of sodium in the acacia-injected rats is not demonstrated by the

TABLE I  
Changes in Serum and Muscle Electrolyte and Muscle Glycogen in Rats  
Average results plus or minus the standard errors are given.

Average results plus or minus the standard errors are given.													
	Experimental group	No. of rats	Serum				Muscle per 100 gm. fat-free solids						
			Per liter serum ultrafiltrate		Per liter serum K	H <sub>2</sub> O percent	Na mm	Cl mm	P mm	K mm	N gm.	H <sub>2</sub> O cc.	Glycogen gm.
			Na mm	Cl mm									
Fed rats	I. Controls	12	144.9 ±0.94	110.8 ±1.24	4.0* ±0.21	94.04 ±0.18	8.8 ±0.39	6.0 ±0.19	32.4 ±0.37	48.6 ±0.24	15.38 ±0.14	340.1 ±1.52	2.09 (8)† ±0.17
	II. Glucose-injected 4½-5½ hrs.†	10	139.4 ±1.20	102.5 ±1.56	6.3 ±0.33	93.4 ±0.23	9.1 ±0.69	5.9 ±0.40	34.2 ±0.35	51.4 ±0.61	15.80 ±0.11	353.7 ±4.21	
	III. Acacia-injected 4-5 hrs.†	14	144.4 ±0.45	99.4 ±2.18	5.9 ±0.52	94.08 ±0.14	7.8 ±0.23	5.4 ±0.21	34.4 ±0.17	50.4 ±0.50	15.79 ±0.08	345.3 ±2.77	2.41 (8)† ±0.11
	IV. Scalded 1-16 hrs.†	15	142.5 ±1.00	114.3 ±0.62	6.8 ±0.33	94.44 ±0.11	8.7 ±0.18	5.8 ±0.16	33.5 ±0.19	50.4 ±0.36	15.70 ±0.08	346.8 ±1.98	1.98 (6)† ±0.18
	V. Epinephrine 3-4 hrs.†	6	140 ±0.45	108.5 ±1.24		94.34 ±0.27	10.0 ±0.10	6.8 ±0.17	35.3 ±0.56	51.6 ±0.51	15.46 ±0.26	354 ±2.10	1.29 (3)† ±0.11
Fasted rats	I. Controls	11	144 ±0.95	111.9 ±1.39	4.6 ±0.15	94.1 ±0.17	9.2 ±0.13	6.5 ±0.26	33.6 ±0.25	47.2 ±0.30	15.4 ±0.07	343.7 ±2.38	1.96 ±0.14
	II. Scalded 1-6 hrs.†	8	140.8 ±1.77	114.1 ±1.21	5.7 ±0.66	94.9 ±0.12	9.3 ±0.38	7.1 ±0.26	34.6 ±0.41	49.0 ±0.61	15.4 ±0.20	342.7 ±1.50	1.61 ±0.09
	III. Epinephrine 3-4 hrs.†	9	139.4 ±1.00	112.3 ±2.30	5.1 ±0.18	94.1 ±0.15	10.2 ±0.44	8.1 ±0.40	35.0 ±0.52	50.0 ±0.74	15.5 ±0.09	354.4 ±2.70	0.72 ±0.14

\* Taken from a previous study (5).

† The number of rats on which glycogen determinations were made.

‡ Range of duration of experiments.

analysis of electrolyte in the serum. From sodium and chloride determinations of the fluid removed from the peritoneum of the acacia-injected rats it was estimated that about 18 per cent of the total available extracellular electrolyte had been removed. In the glucose-injected rats it was calculated that about 23 per cent of the extracellular fluid had been removed as a result of the intraperitoneal injection. The failure to lower the concentration of sodium in the serum of the acacia-injected rats probably is the result of the removal of a smaller amount of extracellular electrolyte by way of the peritoneal fluid and the greater output of urine by these rats. The increase in muscle water in the rats receiving intraperitoneal glucose is largely the result of a shift of water into the intracellular space. Evidence of sodium loss is not present in the data on the scalded rats. However, examination of the scalded area of the skin showed that appreciable amounts of fluid had been immobilized in the involved area. Congestion and induration of the subcutaneous tissue in the scalded zone were readily apparent. The changes in water and extracellular electrolyte in the epinephrine-injected rats are interesting. The extracellular water of the muscle is increased, as denoted by a significant rise in muscle sodium and water and a tendency for the muscle chloride to be increased. The concentration of sodium in the serum is decreased. The explanation of the decrease in serum sodium and the apparent increase in extracellular muscle water is not possible from the data at hand.

The concentration of potassium in the serum is increased significantly in the fed rats in Groups II to IV in Table I. Potassium determinations in the serum of the epinephrine-injected rats in Group V were made in only two of the six animals. Both results were 6.0 milliequivalents per liter of serum, levels that are comparable to those found in the other experimental groups of fed rats in Table I.

The striking increase in potassium, phosphorus, and nitrogen in the muscle of the first few experimental rats suggested the possibility that some solid substance was leaving the muscle cell or being oxidized. Loss of organic solids from the cell could cause an increase of potassium, phosphorus, and nitrogen, provided that these elements were not carried out of the cell immediately. Changes in muscle glycogen were sought for, as this solid was known to be considerably reduced in adrenal insufficiency, which is also characterized by increased concentrations of serum and muscle potassium (6). No reduction in the glycogen content of the muscle was found in the fed rats of Table I except in those injected with epinephrine. Even in the latter rats the decrease in glycogen could account for only about one-fifth of the rise in potassium and phosphorus in the muscle.

The experiments with scalding and the injection of epinephrine were repeated on fasted animals in order to study the electrolyte changes in the

muscle when the glycogen content of the muscle was more uniform from animal to animal. The data on the fasted rats in Table I amply confirm the observations on the fed rats in every respect. The potassium and phosphorus are increased in the muscle of the scalded rats and of those receiving epinephrine and the muscle glycogen is decreased in the latter. The similarity in the glycogen content of the muscle of the fed and fasted control rats probably is related to the fact that the fed rats were not sacrificed until mid-afternoon, some 10 or 12 hours after the usual time of eating. The decrease in the sodium concentration of the serum of the epinephrine-injected rats that had been fasted in the presence of an apparent increase in extracellular water of the muscle is similar to the results on the corresponding group of rats that had been fed (Table I).

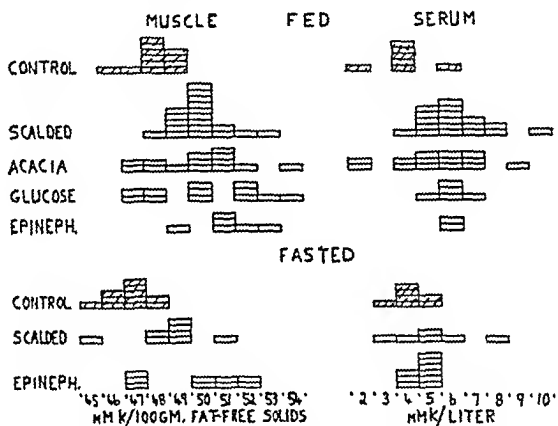


FIG. 1. Serum and muscle K in fed and fasted rats

An interesting incidental observation was the significant decrease in muscle potassium and increase in muscle phosphorus in the fasted control rats when compared to the fed control rats in Table I.

The statistical significance of the increase in muscle and serum potassium in the experimental animals in Table I is apparent. The statistical analysis, however, does not bring out clearly the fact that these increases are not consistently obtained from animal to animal. The levels of muscle and serum potassium of the individual rats in Table I have been shown graphically in Fig. 1 to illustrate that in almost one-third of the experiments concentrations of muscle and serum potassium were obtained which fall within the normal range. In a small series of experiments the change in serum and muscle potassium might be missed.

The significant increase in potassium per 100 gm. of fat-free solids shown in Table I suggested that potassium might be entering the muscle from other sources, possibly the liver or from the gastrointestinal contents. Such a course of events would not explain the increases in phosphorus and nitrogen in the muscle, unless phosphorus and nitrogen also were found to be entering the muscle cells. Although the suggestion of potassium shift from other tissues appeared unlikely, the livers and gastrointestinal tracts with their contents were analyzed in some of the glucose- and acacia-injected rats to test this possibility. The results are given in Table II. It is seen that the livers of the sodium-depleted rats are practically the same in all respects as the livers of the control animals. The slight disparity between the amount of potassium in the small intestinal tract of the nor-

TABLE II

*Electrolytes in Livers and Gastrointestinal Tract Plus Contents of Fed Normal and Sodium-Depleted Rats*

Concentrations in the liver represent the mean result plus or minus the standard error.

	No. of rats	Livers per 100 gm. fat-free solids						No. of rats	Small bowel and contents	Large bowel and contents
		Na	Cl	K	P	N	H <sub>2</sub> O		Total potassium	
		mm	mm	mm	mm	gm.	cc.		mm	mm
Normal*	10	10.4 ±1.3	11.0 ±0.7	35.9 ±2.3	40.5 ±2.7	12.7 ±0.6	290 ±7.5	3	1.23	0.53
Sodium-depleted	9	9.1 ±0.5	10.6 ±0.4	35.6 ±0.86	41.2 ±1.2	13.3 ±0.3	292 ±8.3	3	0.97	0.52

\* Taken from a previous study (7).

mal and the sodium-depleted rats is not great enough by two-thirds to account for the rise in muscle potassium of the latter animals.

The brief span of 3 to 5 hours during which most of the experiments were conducted did not seem to permit sufficient time to produce potassium retention as a result of an impaired renal excretion of this cation. To confirm this impression urine was collected from two rats receiving glucose and four rats injected with acacia. The amount of urine and its content of sodium, chloride, and potassium were determined for each of the six rats over the whole of the experimental period, which lasted about 4½ hours in each animal. The acacia-injected rats voided up to 10 cc. of urine and excreted potassium in dilute concentrations in amounts up to 0.1 mm. The glucose-injected rats voided smaller amounts of urine but greater quantities of potassium and in concentrations as high as 75 mm per liter. The concentrations of potassium in the muscle did not fall below 50 mm

per 100 gm. of fat-free solids in any rat despite the considerable output of urine and potassium. Serum potassium concentrations varied from 5.5 to 7.0 mm per liter of serum.

#### DISCUSSION

Two concepts concerning the regulation of potassium in the serum and muscle can be formulated from the experimental evidence now available. Neither concept excludes the other. The first concept is based on previous investigations. These have amply demonstrated that the concentration of potassium within the muscle cell reflects changes in the concentration of potassium in the extracellular fluid, the organic composition of the muscle remaining approximately constant. Alterations in the potassium levels of the extracellular fluid are usually mediated in turn through changes in renal excretion of this cation. When the concentration of potassium in the serum is increased as the result of total nephrectomy, ureteral ligation, adrenal insufficiency, or by means of injection of potassium salts, the concentration of potassium in the muscle cell is increased (5, 6, 8). Conversely, decreasing the concentration of potassium in the serum by injecting desoxycorticosterone acetate or by feeding low potassium diets produces a fall in the concentration of potassium in the muscle cell (9-11). These changes are likewise mediated through the kidney (12).

The concept of potassium regulation in serum and muscle by means of the kidney cannot be used to explain the increases in muscle potassium observed in the present study. The short duration of the experimental period, the large output of urine noted in some animals, and the failure to find extramuscular sources of potassium in the present study make it necessary to establish a new concept of potassium regulation. The experiments reported here clearly indicate that a change in some fat-free solid has occurred. Since the concentrations in the muscle in the present study are expressed per unit of fat-free solids, a change in some fat-free solid would be reflected in the apparent concentrations of the other substances. The increases in potassium, phosphorus, and nitrogen of the muscle in the sodium-depleted and scalded rats that had been fed (Table I) amount to 4 and 5 per cent per unit of solids. Hence, the loss of some solid equal to about 4 or 5 per cent could explain the changes demonstrated in the muscle. If calculated on a wet tissue basis, the loss of substance amounts to about 0.7 gm. per cent. The nature of the organic substance that is lost is not demonstrated in the data. Glycogen, which is present in the muscle to the extent of about 0.5 gm. per cent, is ruled out by the experiments. Products of lipid metabolism which are soluble in petroleum ether are also eliminated. The possibility that there is some nitrogenous substance or some carbohydrate other than glycogen remains unexplored. The failure

to find that the nitrogen consistently increased in the several experimental groups of animals in Table I may seem to be a serious exception to the concept that some single organic substance is being lost from the muscle. The tendency of the nitrogen to be increased in the fed, scalded rats of Table I might have been significant had a more severe type of burn been used. The change of fed animals from a primarily carbohydrate metabolism to a protein and fat metabolism when fasted might explain the lack of increase in the nitrogen of the scalded, fasted rats in Table I. So far as the epinephrine-injected rats are concerned, Cori and Cori have shown that carbohydrate metabolism is not accelerated following the injection of epinephrine (2) and Reid has shown that an increase in sulfur excretion occurs over 5 and 6 hour periods following the injection of epinephrine in fasted cats and dogs (13).

The rise in serum potassium following burns, intestinal obstruction, and other shock-producing injuries has been noted by many investigators. The work has been reviewed by Seudder (14) and Fenn (15) recently. The latter ascribed the phenomenon to the withdrawal of potassium from body cells. The present experiments give some insight into the nature of this cellular loss. The increase in muscle potassium following sodium depletion and scalds may be reduced to normal concentrations by a resynthesis of whatever organic compound is lost from the cell or by a movement of potassium out of the muscle cell. The fact that muscle potassium remains elevated for several hours suggests a delay in the resynthesis of the organic matter and offers ample opportunity for potassium to escape from the cell to the extracellular fluid. It is surprising that potassium readjustment by this means is not made more rapidly, but it is fortunate for the organism that such is not the case. The return of muscle potassium from 51 mm per 100 gm. of fat-free solids to a normal level of 49 mm would release enough potassium in a 300 gm. rat to raise the serum level to 15 mm if it were confined to the extracellular fluid or to 7.5 mm if distributed throughout the whole body water. Inasmuch as the increase in muscle potassium has been demonstrated to occur without any apparent increase in intracellular water, there should be a steep osmotic gradient from cellular to extracellular fluid. The highest serum potassium concentration in any of the experimental rats in this study was 10 mm per liter in a scalded rat. This is just about the level at which toxic effects on the heart may be expected (16). The conclusion that potassium may be released from muscle as the result of a remote injury and thus secondarily affect cells in other organs of the body is given some support by these studies.

The nature of the stimulus required to set off the course of events that will lead to a loss of organic substance from the muscle cell and a relative increase in potassium, phosphorus, and sometimes nitrogen in the muscle

has not been clearly defined by the present investigation. While some degree of depletion or immobilization of extracellular fluid or decrease in serum sodium concentration was present in each experiment, it is not certain that this is the sole type of injury that will produce the results obtained here. Other types of injury have not been tried.

#### SUMMARY

Potassium, phosphorus, and sometimes nitrogen are relatively increased in the muscle cell following the partial depletion or immobilization of extracellular electrolyte. These changes occur in the rat within a few hours of the injury and are dependent on a loss of some as yet unidentified organic substance from the muscle cell and are independent of any action of the kidneys. The alterations in muscle electrolyte are accompanied by an increase in concentration of potassium in the serum.

This study suggests that the rise in serum potassium observed clinically in burns and other shock-producing injuries may be related to the loss of organic substance from the muscle cell which accompanies depletion or immobilization of extracellular fluid.

*Addendum*—Since the completion of this study Clarke and Cleghorn (17) have reported increases in the concentrations of serum potassium and muscle potassium and phosphorus within 2 to 6 hours following bodily injury in rats and muscle trauma in dogs. Their results are analogous to those presented here.

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## PARTIAL OXIDATION OF CHOLIC ACID\*

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The behavior of polyhydroxy steroids toward oxidizing agents has been studied extensively in the bile acids. Wieland and Dane (1) originally suggested that the order in which the hydroxyl groups of cholic acid are dehydrogenated is 7-3-12 (older literature, 12-3-7). This was based upon their claim that oxidation of desoxycholic acid yielded 3-keto-12-hydroxy-cholanic acid, while 7,12-dihydroxycholanic acid (probably a mixture of isomers) was oxidized by  $\text{CrO}_3$  to 7-keto-12-hydroxycholanic acid. The same investigators (2) proved that the 6-hydroxyl of hyodesoxycholic acid was more readily attacked by  $\text{CrO}_3$ , since low temperature oxidation gave the 6-keto-3-hydroxy compound. Iwasaki (3) prepared 3-hydroxy-7-ketocholanic acid from both chenodesoxycholic acid and ursodesoxycholic acid by chromic acid oxidation at low temperature in agreement with the Wieland and Dane suggestion.

In 1937 Kaziro and Shimada (4) proved that the hydroxyl groups of the bile acids were not oxidized in the order 7-3-12, since partial oxidation of desoxycholic acid by  $\text{CrO}_3$  yielded 3-hydroxy-12-ketocholanic acid rather than the expected 3-keto-12-hydroxy acid. When these authors investigated the partial oxidation of cholic acid, they obtained in 65 per cent yield 3-hydroxy-7,12-diketocholanic acid ("reductodehydrocholic acid"). Under a variety of conditions they were unable to obtain any monoketo acid; when 3-keto-7,12-dihydroxycholanic acid was oxidized, the sole product obtained was dehydrocholic acid. They confirmed the considerable difference in the oxidation of the 3-OH compared with those at  $\text{C}_7$  and  $\text{C}_{12}$  and concluded that these latter were almost identical in the ease with which they were oxidized by chromic acid.

It seemed to us that there must be a significant difference in the ease of oxidation of the  $\text{C}_7$  and  $\text{C}_{12}$  hydroxyl groups of cholic acid and that the results of Kaziro and Shimada were to be explained by their failure to isolate the 7-ketocholic acid. This view is probable, since the reductodehydrocholic acid which they obtained exhibits an unsatisfactory melting

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point behavior, so that an impurity might not easily be recognized. We therefore studied the low temperature oxidation of cholic acid and the methyl ester; we separated the reaction products by conversion to the acetates of the methyl esters and found that these could be readily purified by chromatographic adsorption on aluminum oxide. We obtained the hitherto undescribed 7-keto-3,12-dihydroxycholan-ic acid in 40 per cent yield. These experiments then demonstrate that in cholic acid the hydroxyl group at C<sub>7</sub> is most readily attacked by CrO<sub>3</sub> and at the same time offer a method by which desoxycholic acid may be prepared from the much more abundant cholic acid. Haslewood (5) has independently reported a similar conclusion.

#### EXPERIMENTAL

*Oxidation of Methyl Cholate*—10 gm. of methyl cholate (m.p. 145–147°) were dissolved in 300 cc. of stable glacial acetic acid and 50 cc. of water. The solution was cooled to -7° and 81.5 cc. of 3.5 N CrO<sub>3</sub> in 25 per cent acetic acid (3 equivalents) were added dropwise over 4 hours, so that the temperature never rose over 0°. The reaction mixture was stored overnight at 0°, sodium bisulfite was added in excess, and the mixture evaporated on the steam bath under reduced pressure. The residue was dissolved in ethyl acetate, washed with water, dilute acid, sodium bicarbonate solution, and again with water. After drying over anhydrous sodium sulfate the solution was evaporated and the residue weighed 38.7 gm.

*Separation of Oxidation Mixture*—The oily residue was acetylated by heating with acetic anhydride in pyridine for 22 hours. The acetates were dissolved in 3.5 liters of 90–100° petroleum ether and poured over a column of 350 gm. of aluminum oxide (Aloreco brand, C-30 grade) which had been activated by heating for 30 minutes at about 450°. The percolating fluid was collected in liter portions and the washing continued with 90–100° petroleum ether until no significant amount of solid was eluted. This required 12 liters and 20 gm. of solid were eluted. This material was crystallized by rubbing with a small amount of 90–100° petroleum ether. After three crystallizations from the same solvent, the product was obtained as long prisms melting at 114–117° corrected,  $[\alpha]_D^{25} = +64^\circ$  (absolute ethanol), and proved to be 3,12-diacetoxy-7-ketocholan-ic methyl ester.<sup>1</sup>

C<sub>29</sub>H<sub>44</sub>O<sub>7</sub>. Calculated, C 69.00, H 8.79; found, C 69.08, H 8.69

The oxime was prepared by boiling a solution of 490 mg. in ethanol with 600 mg. of sodium acetate and 500 mg. of hydroxylamine hydrochloride

<sup>1</sup> All analyses were made by Dr. T. S. Ma of the Department of Chemistry, University of Chicago.

for 3 hours. The product crystallized from methanol in long needles, m.p. 155.5–157° corrected.

$C_{22}H_{43}O_7N$ . Calculated. C 67.01, H 8.51, N 2.70  
Found. " 66.99, " 8.73, " 3.00

The 3-acetoxy-7,12-diketocholelanic methyl ester was removed from the column by elution with 90–100° petroleum ether containing 2 per cent ethyl acetate. The methyl ester of dehydrocholic acid could finally be removed with ethyl acetate alone. In general about 40 per cent of the monoketo, 40 per cent of the diketo, and 20 per cent of the triketo compounds were obtained.

*7-Keto-8,12-dihydroxycholelanic Acid*—10.2 gm. of 7-keto-3,12-diacetoxymethyl cholanate (m.p. 112–114°) were dissolved in 50 cc. of 95 per cent ethanol, 75 cc. of 1.7 N aqueous NaOH added, and the mixture heated under a reflux for 4 hours. After neutralization to phenolphthalein, 66.8 cc. of N base were neutralized; calculated for 3 equivalents 66.5 cc. of N base. Most of the alcohol was distilled and the aqueous solution added dropwise with stirring to dilute  $H_2SO_4$  at 50°. The acid crystallized on cooling and was recrystallized from ethyl acetate as well formed prisms, m.p. 197–199° (corrected), with a negligible rotation in absolute ethanol.

$C_{21}H_{41}O_6$ . Calculated, C 70.88, H 9.43; found, C 70.54, H 9.36

The methyl ester prepared with diazomethane crystallized from ethyl acetate-petroleum ether in small needles, m.p. 152–154°.

$C_{22}H_{43}O_6$ . Calculated, C 71.37, H 9.59; found, C 71.35, H 9.75

*Preparation of Desoxycholic Acid*—The semicarbazone of the 7-keto-3,12-diacetoxymethyl cholanate was prepared by heating 1.0 gm. of ester with 1.27 gm. of semicarbazide acetate in a mixture of 40 cc. of absolute methanol, 20 cc. of pyridine, and 5 cc. of water under a reflux for 6 hours. The product was well washed with boiling water and crystallized from ethanol. Needles, m.p. 175–177° (corrected).

$C_{20}H_{37}O_7N_2$ . Calculated, N 7.48; found, N 7.78

440 mg. of the semicarbazone were heated with 6 cc. of absolute alcohol containing 350 mg. of sodium and 0.6 cc. of hydrazine hydrate in a sealed tube at 200° for 8 hours. The reaction mixture was acidified and a white semicrystalline product separated; weight 280 mg. The material crystallized well in clumps of narrow prisms from dilute acetic acid; weight 216 mg., m.p. 139–143° (corrected). The melting point of a mixture with desoxycholic-acetocholeic acid was 139–142°. The diformyl derivative was prepared by warming at 55° for 5 hours with 88.5 per cent formic acid

and crystallized from dilute ethanol, m.p. 192.5–191°. Cortese and Bauman (6) record a melting point of 193–194°.

#### SUMMARY

1. The hydroxyl groups of cholic acid are oxidized by  $\text{CrO}_3$  in acetic acid in the order, 7–12–3.
2. A method for the separation of 7-keto-3,12-dihydroxycholan-ic acid from the oxidation mixture is described.
3. This compound can be reduced to desoxycholic acid in good over-all yield from cholic acid.

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# THE ENZYMATIC NATURE OF ANGIOTONIN FORMATION FROM RENIN AND RENIN ACTIVATOR

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Renal hypertension is believed to be caused by an alteration in a vasopressor system having its origin in the kidneys (1). Renin, liberated from the kidneys, reacts with a globulin fraction from the blood (renin activator) to produce a pressor substance, angiotonin (2, 3). Renin itself is not vasopressor nor do any of the blood globulins exhibit pressor activity. Angiotonin is physiologically highly active but can be inactivated by angiotonase, an enzyme present in the kidneys and other tissue (4). The formation and destruction of angiotonin are possibly balanced in normal animals and, when the balance is disturbed, hypo- or hypertension may occur. Although the reactions are intimately connected, they can be studied separately.

It has been suggested that the reaction is enzymatic (5, 6), renin acting as the enzyme and renin activator as the substrate, but rigorous proof is lacking. This investigation is concerned with the kinetics of angiotonin formation in an attempt to show that its rate is first order, a necessary demonstration before its enzymatic nature can be taken as anything more than a suggestion.

## EXPERIMENTAL

The renin used in the experiments described below was prepared according to Helmer and Page (7). The angiotonase was removed from the product by adjusting the renin solution to pH 2.0 and precipitating with 30 gm. of sodium chloride per 100 cc. of solution. The precipitate was collected on a filter, dissolved in sufficient water to make approximately a 2 per cent solution, and dialyzed against running tap water. For final dialysis, cold toluene-saturated, distilled water was used. The solution was almost colorless and was found to retain its enzymatic activity for at least 2 weeks when kept in the ice box. When incubated with a known amount of angiotonin at 37° for 30 minutes, no reduction of pressor activity occurred. It was therefore free of angiotonase.

The renin activator was prepared from hog serum by fractional precipitation with ammonium sulfate at pH 6.5, the fraction between 1.22 and 2.10 M being collected. With the aid of the rotating cellophane bag technique

TABLE I

*Angiotonin Responses Elicited by Incubation of Same Amount of Activator (5 Cc., 2 Per Cent Protein) with Various Amounts of Renin*

The results are expressed in terms of an arbitrary unit defined as  
Mm. blood pressure rise of 1 cc. test sample

Mm. blood pressure rise of 0.1 cc. standard angiotonin

Renin A	Experiment I	Renin B	Experiment II
cc.	angiotonin unit	cc.	angiotonin unit
0.001	0.10	0.001*	0.14
0.002	0.14	0.01	0.29
0.003	0.26	0.05	0.48
0.007	0.40	0.5	0.63
0.5	0.50	2.0	0.64
1.0	0.52	2.0†	0.59

\* Incubated at 25° for 10 minutes.

† Incubated at 25° for 5 hours.

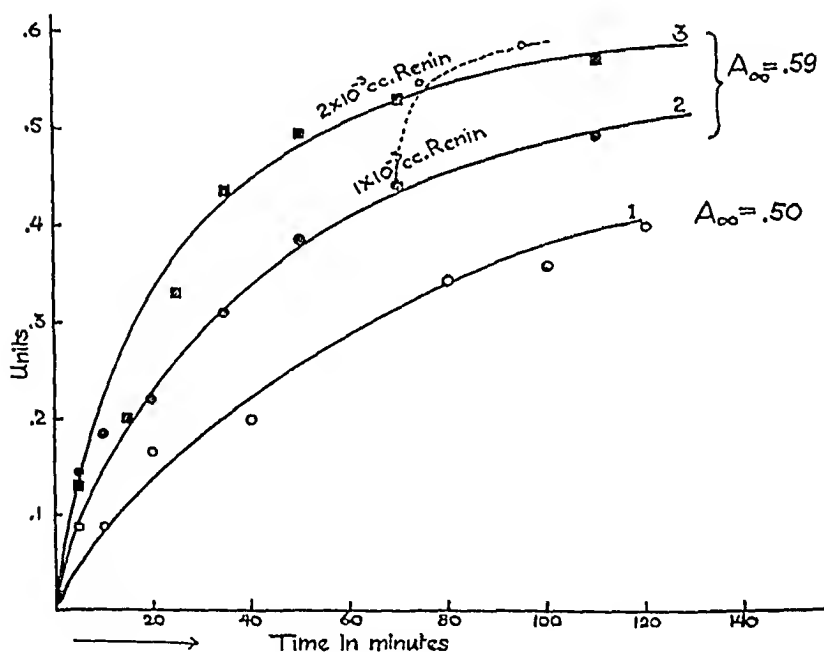


FIG. 1. Angiotonin pressor responses as a function of incubation time. Curve 1 was obtained by incubation at 37°, and Curves 2 and 3 were prepared by incubation at 25°. The concentration of renin in experiments leading to Curve 3 was twice as great as the ones for Curve 2.  $A_{\infty}$  represents maximum angiotonin responses characteristic of the particular activator employed. These infinity values were obtained by incubation with excess renin. The dotted line represents the response on addition of a second portion of renin.

(8) at room temperature all pigments were eliminated in the last filtrate at 2.10 M ammonium sulfate. The cuglobulin was eliminated by prolonged dialysis against running tap water and finally against distilled water. The final product was a clear solution with slight blue fluorescence. Electrophoretic analysis of a 1 per cent solution in phosphate buffer at pH 7.62 indicated 18 per cent albumin, 56 per cent  $\alpha$ -globulin, 6 per cent  $\beta$ -globulin,

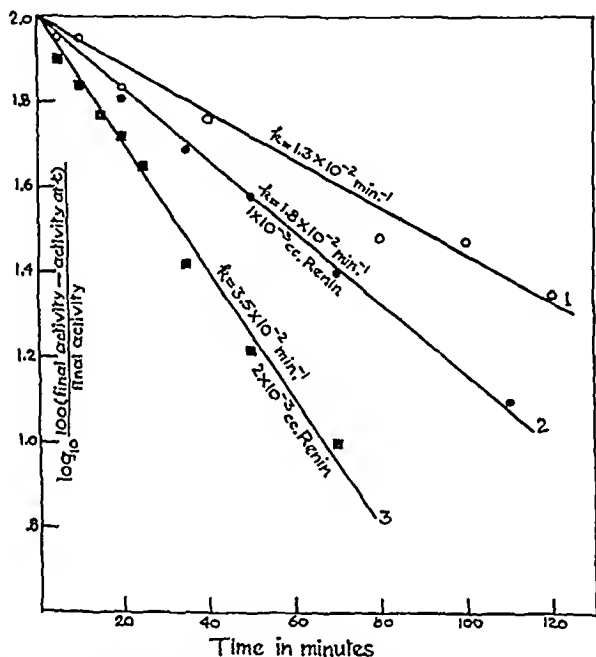


FIG. 2. Logarithm of substrate concentration plotted against time. Curves 1, 2, and 3 correspond to Curves 1, 2, and 3 of Fig. 1.

and 23 per cent  $\gamma$ -globulin. We are greatly indebted to Dr. Wm. W. Davis of the Lilly Research Laboratories for this analysis.

**Incubation**—Renin activator (5 cc.) was incubated at 37° for 10 minutes with various amounts of renin solution. The mixture was then brought to pH 5.5 by addition of a few drops of 1 per cent hydrochloric acid and immediately placed in a boiling water bath. The pH of optimum coagulation was found to be between 5.3 and 5.8. After being boiled for 10 minutes the solutions were centrifuged or filtered through coarse paper.

**Biological Testing**—1.0 cc. of the filtrate was injected into the femoral



vein of a pithed cat.<sup>1</sup> The resulting rise in blood pressure was compared to the rise obtained from 0.1 cc. of a standard angiotonin preparation equivalent to 3.3  $\gamma$  of epinephrine. Since the responses of the standard angiotonin were found to be constant for two or three successive injections only, testing of the incubation mixture was always followed by injection of 0.1 cc. of standard angiotonin. Angiotonin units are given as the ratio

$$\frac{\text{Mm. blood pressure rise of test sample}}{\text{Mm. blood pressure rise with 0.1 cc. angiotonin}}$$

When the angiotonin pressor responses are plotted against cc. of renin, the resulting curve is characteristic of a first order reaction. The results obtained with two different renin samples are given in Table I.

Next, the relation of the incubation time to angiotonin formation was investigated. 5 cc. of renin activator were incubated with an amount of renin calculated to give about one-fifth the maximum angiotonin response after 10 minutes incubation. The technique employed in the incubation, denaturation, and method of testing of the resulting angiotonin solution was the same as described above.<sup>2</sup> The maximum amount of angiotonin which this particular activator preparation was capable of yielding was determined by incubation of the same amount of renin activator with a large excess of renin ( $A_{\infty}$  in Figs. 1 and 2). Theoretically this value should be determined by incubation for a prolonged period. With extremely small amounts of renin (Table I; Fig. 2, Curve 1) 5 to 10 hours incubation would be necessary before the maximum amount of angiotonin could be obtained. Although the activator employed in these experiments was almost free of inhibitor, 5 or 10 hours incubation at 37° was found to destroy 10 to 15 per cent of the angiotonin. This may be due to a small amount of inhibitor, instability of the product (angiotonin), or, most probably, to bacterial action.

Curve 2, Fig. 1, represents angiotonin response as a function of incubation time. At the point indicated a further quantity of renin was added, which increased markedly the amount of angiotonin formed per unit of time. The maximum amount of angiotonin was formed within 25 minutes after the addition of the second portion of renin.

#### DISCUSSION

Page and Helmer (3) suggested that renin is an enzyme, because it is a heat-labile protein, its reaction with activator is slow and affected by temperature, and only small amounts are required relative to activator.

<sup>1</sup> We wish to thank Dr. K. G. Kohlstaedt and Mr. C. Wilson for the animal assay.

<sup>2</sup> Curve 1 (Figs. 1 and 2) was obtained by incubation at 37°, while Curves 2 and 3 are the result of incubations at 25°.

In addition to these arguments, Muñoz, Braun-Menendez, Fasciolo, and Leloir (9) pointed out that for a given amount of activator the maximum yield of angiotonin cannot be surpassed when a 10 or 20 times excess of renin is used. These authors explained that, "if the reaction were stoichiometric, the maximum amount of hypertensin [angiotonin] formed ought to be proportional to the amount of renin."

Since neither of the two reactants (renin and renin activator) was available in pure form, it seems questionable whether the reasons given in the literature can be accepted as sufficient proof for the enzymatic nature of the reaction. Muñoz, Braun-Menendez, Fasciolo, and Leloir used serum as renin activator, while Page and Helmer employed a globulin fraction precipitated between 0.33 and 0.55 saturated ammonium sulfate. Recent studies of serum globulins indicate that they consist of a number of chemically distinct proteins, any one of which may be identical with the renin activator. Consequently even if the total amount of protein in the renin activator solutions were known, no conclusions can be drawn concerning the concentration of the "substrate." Similar arguments apply to the quality and quantity of renin. Since no analytical method for the determination of renin was available, the quantity of renin used by these investigators was expressed in relative units; *i.e.*, cc. of renin solution or an arbitrary unit defined in terms of pressor response of a third substance (angiotonin) (10-12).<sup>3</sup> It is probable that the major constituent of renin activator and renin solutions is a mixture of inert proteins and that the active principles are present in extremely small amounts. The relation of the maximum amounts of product (angiotonin) formed to the amount of renin employed has little or no bearing upon the nature of the reaction and surely none as regards its order. To state with certainty whether renin reacts stoichiometrically with a certain globulin fraction can only be answered by determining the rate of the reaction. A first order reaction would indicate an enzymatic process, while a second or third order reaction would necessitate a stoichiometric reaction.

The previously reported studies of this reaction (3, 6) do not lend themselves to such an analysis. In crude preparations of renin and renin activator, substances are present which destroy the freshly formed angiotonin (6). This results in two or more competitive reactions the complexity of which may be too great for a kinetic analysis. Both renin and renin activator used in this investigation were free of angiotonase and this forced

<sup>3</sup> Many attempts have been made to standardize renin solutions. All except one of the proposed methods were based upon the pressor response elicited by intravenous injection of given amounts of renin solutions. Since renin is an enzyme, as this kinetic investigation has shown, it must be tested as an enzyme; *i.e.*, in its catalytic action and not as a pressor substance.

the reaction to proceed in one direction only. Muñoz, Braun-Menendez, Fasciolo, and Leloir (9) reported curves in essence similar to Fig. 1, using angiotonase-free renin and renin activator, but the experimental error involved is somewhat too great for a kinetic study.

Enzymes are organic catalysts and as such are governed by the same laws as are inorganic catalysts. Hence, the amount of substrate decomposed must be proportional at any time to the concentration of the substrate. If  $K$ , represents the reaction constant for a given amount of enzyme and  $S$  the substrate concentration,  $-dS/dt = K.S$ . That is, it must conform with the laws of a first order reaction. When the logarithm of the substrate concentration at time  $t$ , which is proportional to the logarithm of the ratio of the final activity minus the activity at  $t$  to the final activity, is plotted against the time, a straight line is obtained in complete agreement with the requirements of the above equation. A graphic representation of this relationship is given in Fig. 2. The slopes of the curves are proportional to the concentration of renin present, showing that the first order reaction constant is proportional to the renin concentration.

The same conclusions can be drawn from Curves 2 and 3, Fig. 2. Since the renin activator becomes exhausted more rapidly after the concentration of renin is increased, the rate of the reaction is proportional to the concentration of renin.

The authors are indebted to Mr. Fredric R. Van Abeele for his valued assistance.

#### SUMMARY

The reaction of angiotonase-free renin and renin activator was studied from a kinetic point of view. The minimum amount of renin required to produce maximum angiotonin pressor response for the activator employed was determined. Approximately one-fourth of this amount was incubated with renin activator for various lengths of time. When the logarithm of the concentration of renin activator was plotted as a function of angiotonin pressor response against time, a straight line was obtained as required by theory for an enzymatic reaction (first order). The first order reaction constant for a duplicate experiment with twice the amount of renin was found to increase proportionally. The percentage of renin activator decomposed per unit of time was therefore proportional to the renin concentration and independent of the activator concentration. The reaction is therefore enzymatic; renin is the enzyme and renin activator the substrate.

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## THE NATURE OF RENIN ACTIVATOR

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Kohlstaedt, Helmer, and Page (1, 2) showed that renin itself is not physiologically active but requires the presence of proteins in order to be effective as a pressor agent. They suggested that it is enzyme-like. At first serum was used as substrate (2, 3), but a pseudoglobulin fraction prepared from serum was found to be even more effective. This fraction has been temporarily called "renin activator" because the nature of the reaction was not fully understood. The mechanism of the interaction of renin and renin activator has now been more critically analyzed (4), and its enzymatic nature kinetically demonstrated (first order reaction). Since the percentage of renin activator decomposed per unit time was proportional to the renin concentration, renin is the enzyme and renin activator the substrate.

Since the nature of the reaction had been established, it seemed of interest to investigate and prepare both substrate and enzyme in chemically pure form. This report deals with the chemical nature of renin activator.

Tiselius (5) and other investigators have shown that blood globulins consist of a number of electrophoretically distinct proteins and, since renin activator prepared by a rough fractionation of serum appeared to be a mixture of these, we attempted to find which, if any, of these serum components can act as renin activator. Cohn, McMeekin, Oncley, Newell, and Hughes (6) and Green (7) prepared reasonably pure proteins by ammonium sulfate and alcohol fractionation of horse serum. We have studied and fractionated hog serum by similar methods and found renin activator to be identical with or contained within the  $\alpha_2$ -globulin fraction.

### EXPERIMENTAL

*Fractionation of Serum*—Fresh hog blood was allowed to clot and was drained through cheese-cloth. The filtrate was centrifuged in order to remove residual cells. The supernatant serum was siphoned off and found to be very little hemolyzed. It was diluted with 1 volume of water and fractionally precipitated with ammonium sulfate. The rotating cellophane bag technique was employed unless otherwise stated.

*Fraction I ( $\gamma$ -Globulin)*—16 liters of serum were diluted with an equal volume of water and enough ammonium sulfate added to make the solution

1.25 M. The precipitate was dissolved in the minimum amount of water and dialyzed until free of salt. The solution was made up to 4000 cc., which gave a protein concentration of 7 per cent. 76 cc. of 1 N sulfuric acid and 2300 cc. of water were then added to bring the pH of the solution to 6.5, which was followed by addition of 1600 cc. of saturated ammonium sulfate. This was added through a separatory funnel in such a way that the tip of the funnel extended below the surface of the liquid. The concentration of ammonium sulfate at this point was 1.22 M. The precipitate which was formed was filtered off, dissolved in 1500 cc. of water, and exhaustively dialyzed against distilled water until free from ammonium sulfate. The euglobulin was removed by filtration, dissolved in normal saline, and tested as described below. The pseudoglobulin was precipitated four times at 1.22 M ammonium sulfate at pH 6.5 and the final precipitate dissolved in 600 cc. of water and dialyzed until free of salt. Electrophoretic analysis of a 1.7 per cent solution in phosphate buffer at pH 7.7 showed the product to be relatively pure  $\gamma$ -globulin (Fig. 1, b). About 5 per cent of an impurity moving with a velocity of  $\alpha$ -globulin was present.

*Activator*—The filtrate from  $\gamma$ -globulin was brought to 2.10 M by addition of solid ammonium sulfate through cellophane membranes. The filtrate was set aside for precipitation of albumin and the precipitate dissolved in a sufficient quantity of water to make a 4 to 6 per cent solution. After thorough dialysis and removal of the euglobulin the solution was diluted to 2 per cent protein and a small portion of this fraction used for the incubation experiments described below. An electrophoretic analysis of a 2 per cent solution in phosphate buffer at pH 7.62 indicated its composition to be 18 per cent albumin, 56 per cent  $\alpha_2$ -globulin, 6 per cent  $\beta$ -globulin, and 24 per cent  $\gamma$ -globulin (Fig. 1, c). No separate  $\alpha_1$ -globulin was found.

*Fraction II*—The activator solution containing 2 per cent protein was brought to 1.53 M ammonium sulfate and the precipitate discarded. The ammonium sulfate concentration of the filtrate was then increased to 1.70 M, and the filtrate used for the preparation of  $\alpha$ -globulin and the precipitate dissolved in water and dialyzed until free of salt. The euglobulin was discarded, because it was found to be inactive, and the pseudoglobulin once more precipitated between 1.60 and 1.76 M ammonium sulfate. After repetition of this procedure, the final solution was adjusted to pH 5 and dialyzed until no more precipitate formed. A small amount of euglobulin was formed, which was removed by filtration through a heavy layer of Hyflo Filter-Cel and the filtrate diluted to give a 2 per cent solution. This solution was employed for the incubation experiments described below. Electrophoretic analysis of a 2.1 per cent solution in phosphate buffer at pH 7.68 is given in Table II (Fig. 1, d). Since the resolution at this pH

was not very good, the electrophoretic analysis of this fraction was repeated in veronal buffer at pH 8.49 (Fig. 1, e).

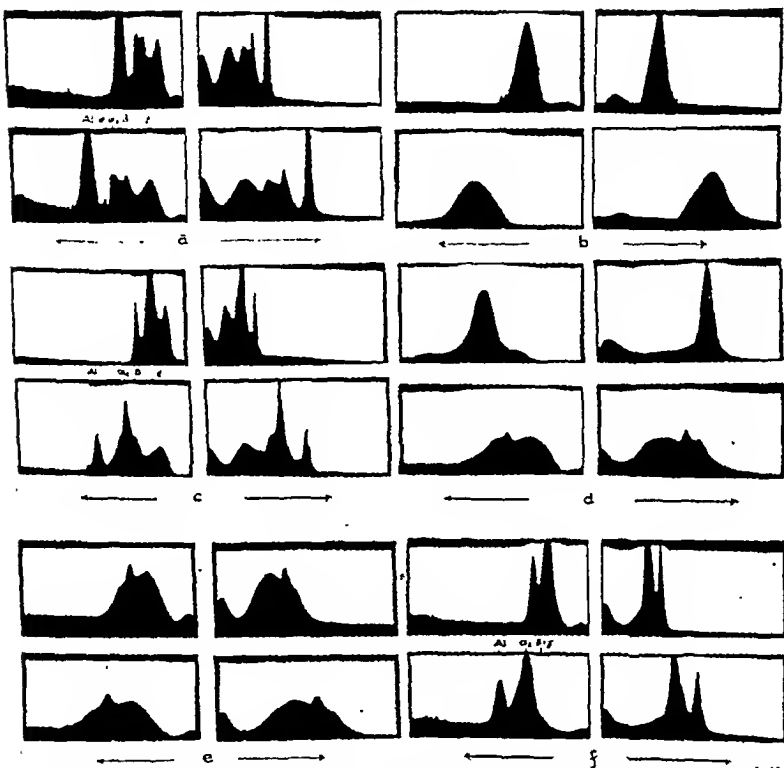


FIG. 1. Electrophoretic patterns of hog serum and fractions precipitated with ammonium sulfate. Patterns with arrows pointing left are descending; right, ascending. (a) Whole serum, upper, 150 minutes; lower, 250 minutes. (b) Fraction I ( $\gamma$ -globulin), upper, 200 minutes; lower, 420 minutes. (c) Activator, upper, 100 minutes; lower, 210 minutes. (d) Upper, Fraction III ( $\alpha$ -globulin), 120 minutes; lower, Fraction II, 220 minutes. (e) Fraction II, upper, 150 minutes; lower, 222 minutes. (f) Fraction IV, upper, 120 minutes; lower, 220 minutes. Veronal buffer (pH 8.49) was employed for Fraction II (e), while all others were made in phosphate buffer (pH 7.7). All exposures were made with Philpot's eyecylinder lens system and a 30° knife-edge diaphragm.

*Fraction III ( $\alpha$ -Globulin)*—The filtrate from Fraction II at 1.70 M was brought to 2.05 M ammonium sulfate, and the precipitate dissolved in water and dialyzed against tap water until free of salt. It was twice



precipitated between 1.85 and 2.10 M ammonium sulfate and the final solution adjusted to pH 5 and treated in the same manner as described for Fraction II. An electrophoretic analysis of a 2.0 per cent solution in phosphate buffer at pH 7.7 indicated 92 per cent  $\alpha_2$ -globulin, 5 per cent of a slower  $\beta$ - or  $\gamma$ -globulin, and 2 per cent of a faster moving component (albumin or  $\alpha_1$ -globulin) (Fig. 1, d).

*Fraction IV*—The filtrate from the activator at 2.10 M ammonium sulfate was brought to 2.56 M, the precipitate collected, dissolved in the minimum

TABLE I

*Preliminary Tests on Globulin and Albumin Fractions*

To 5 cc. of a 2 per cent protein solution were added various amounts of renin solution and the mixture made up to 6 cc. For an explanation of the units see the text. Incubation and testing are described in the experimental portion.

Experiment No.	Substrate	Renin	Units
		cc.	
8	Activator	0.004	0.2
10	"	0.03	0.4
11	"	0.06	0.5
12	"	0.10	0.7
13	"	0.50	0.9
14	Fraction III ( $\alpha$ -globulin)	0.03	0.25
15	" "	0.06	0.4
16	" " "	0.10	0.55
17	" " "	0.50	1.35
18	" II	0.03	0.10
19	" "	0.06	0.0
20	" "	0.10	0.2
21	" "	0.50	0.2
22	" I ( $\gamma$ -globulin)	0.05	0
23	" " "	0.50	0
24	" " "	1.0	0
25	Fraction IV	0.05	0
26	" "	0.50	0
27	" "	1.0	0

amount of water, and dialyzed. When free of salt the solution was adjusted to pH 4.75 by addition of dilute hydrochloric acid and the precipitation repeated twice, any precipitate which formed up to 2.10 M ammonium sulfate being discarded. In the hope of separating albumin, we added saturated ammonium sulfate to incipient cloudiness at pH 7.5, and the protein was precipitated by slow addition of dilute sulfuric acid. An electrophoretic analysis of this isoelectric precipitate in phosphate buffer at pH 7.62 is given in Fig. 1, f, and Table I.

The renin used in this investigation was prepared according to Helmer and Page (8) (alcohol being used instead of acetone to dehydrate the minced

kidneys). This preparation was not free of angiotonase, since 30 minutes incubation of 8 parts of renin with 1 part of angiotonin caused a 60 per cent reduction in pressor activity. Little or no reduction was observed after 10 minutes incubation. Samples of the various globulin and albumin fractions were incubated with specified amounts of renin for 10 minutes, the pH adjusted to 5.5, and the mixture placed in a boiling water bath for 6 to 10 minutes. The denatured proteins were filtered off and 1.0 cc. of the clear filtrate tested in the manner previously described with a pithed

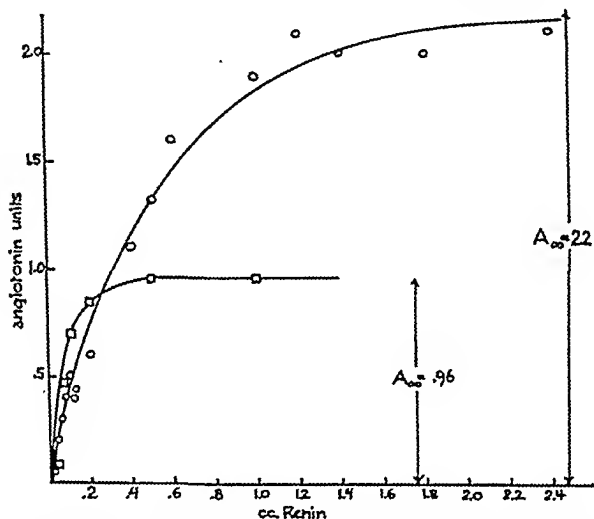


FIG. 2 Comparison of  $\alpha$ -globulin (O) and activator (□) in their efficiency as substrates for angiotonin formation when incubated with increasing amounts of renin. Both substrate solutions contained 2 per cent protein and the same renin preparation was used.  $A_{\infty}$  is the maximum angiotonin response characteristic of the substrate ( $\alpha$ -globulin or activator). These maximum values were obtained by incubation with excessive amounts of renin.

cat as a test animal.<sup>1</sup> The results are expressed in terms of an arbitrary unit defined as the fraction

$$\frac{\text{Mm. blood pressure rise of 1.0 cc. sample}}{\text{Mm. blood pressure rise of 0.1 cc. standard angiotonin}}$$

<sup>1</sup> The pithed cat was found to be the most satisfactory animal for this assay. However, it must be kept in mind that all responses must be below a limiting value characteristic of the animal and its state of health. An animal in which the responses are not additive (doubling the quantity of angiotonin must produce twice the pressor response) is useless for the assay. We wish to thank Dr. K. G. Kohlsteadt and Mr. C. Wilson for the animal assay.

As a preliminary test all fractions were incubated with various amounts of renin at pH 7.0 in order to determine which, if any, of them contains the substrate or can act as such. The results of these tests are given in Table I from which it can be concluded that only the  $\alpha$ -globulin fraction shows

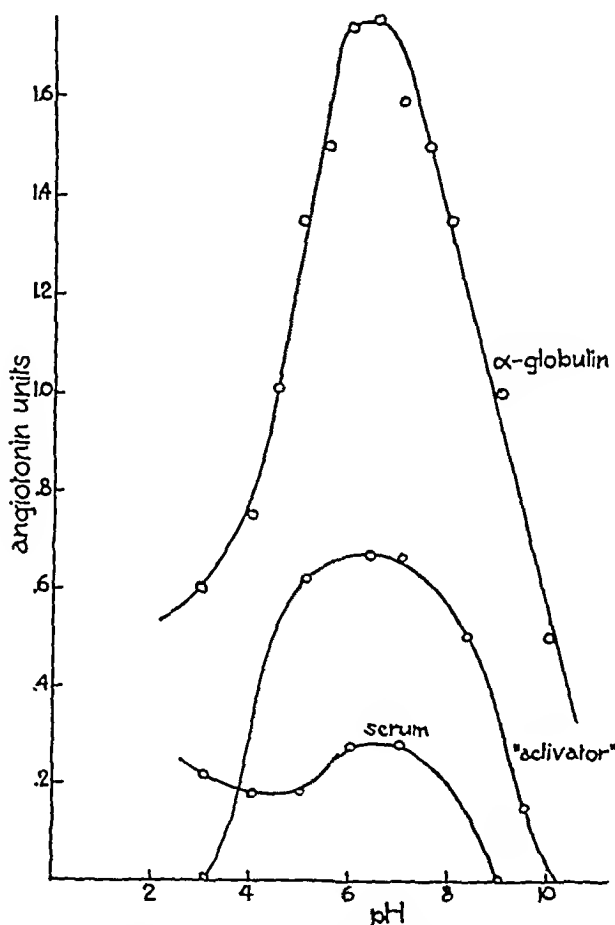


FIG. 3. Comparison of hog serum, activator, and  $\alpha$ -globulin in their efficiency as substrates for angiotonin formation when incubated with renin at various pH values. All substrates contained 2 per cent protein.

any activity.  $\alpha$ -Globulin was therefore studied more extensively, smaller increments of renin being used in order to arrive at a more complete curve which is reproduced in Fig. 2. The maximum amount of angiotonin was not reached in the preliminary tests (Table I), since  $\alpha$ -globulin is capable of producing 2.2 angiotonin units under the condition of the experiment.

Responses elicited by incubation of renin with activator (2 per cent total protein) are plotted for comparison.

Another series of experiments was carried out in order to study the effect of pH on this enzymatic reaction. 5.0 cc. of 2 per cent  $\alpha$ -globulin solution were adjusted to various pH values, 1 cc. of renin solution of the same acidity added, and the volume made up to 7 cc. The mixture was incubated at 37° for 10 minutes and after the pH was adjusted to 5.5 the proteins were denatured by heating and removed by filtration through coarse paper. 1 cc. of this filtrate was used for biological testing.

The same experiments were repeated with activator and fresh hog serum each containing 2 per cent total protein. The results of these experiments are given in Fig. 3.

#### DISCUSSION

The investigations of Cohn, McMeekin, Oncley, Newell, and Hughes (6) and Green (7) had shown that horse serum can be fractionated with ammonium sulfate, giving reasonably pure globulins and crystalline albumins. The order in which these proteins precipitate with ammonium sulfate corresponds to their electrophoretic mobilities. As the salt concentration increases, the globulins precipitate in the order  $\gamma$ -,  $\beta$ -, and  $\alpha$ -globulin, followed by albumins, enzymes, and pigments. If plasma instead of serum is used, fibrinogen represents an exception to this rule: its mobility lies between  $\beta$ - and  $\gamma$ -globulin at pH 7.7; yet it is the first to precipitate upon addition of ammonium sulfate or alcohol.

A comparative study of horse, cow, swine, and rabbit serum has been reported by Svensson (9). Although Svensson did not isolate the protein constituents by repeated fractionation, his investigations indicate that such a separation is possible. Following a suggestion of Tiselius, he analyzed the filtrates after addition of various amounts of ammonium sulfate instead of the precipitates. This method is to be preferred for a preliminary study and his results clearly show that the precipitating power of ammonium sulfate is quite similar with all these sera. Although the percentage of each globulin differs with the species, the order in which they are removed from the solution is the same. Since he precipitated the fractions by addition of solid ammonium sulfate, coprecipitation must be taken into account in any interpretation of his data. This, however, would not essentially change the order of precipitation.

With this in mind we attempted to prepare the various globulin fractions from hog serum using a procedure similar to the one reported by Cohn, McMeekin, Oncley, Newell, and Hughes (6). The limits within which these proteins precipitate were found to be somewhat different from the values reported for horse serum, although the data of Svensson (9) would

indicate that they are almost identical. Thus, globulins were not expected to precipitate above 2.10 M ammonium sulfate and any precipitate above that concentration should be albumin; yet the major constituent of Fraction IV (2.10 to 2.56 M ammonium sulfate) was  $\alpha_2$ -globulin.

Examination of the electrophoretic patterns of whole serum (Fig. 1, a) revealed two distinct  $\alpha$ -peaks, similar to the two forms of  $\alpha$ -globulin in horse serum (9). Since their electrophoretic mobilities are distinctly different, they are probably chemically non-identical. Tiselius (5) reported only one  $\alpha$  component in horse serum and Svensson believes that the double nature of  $\alpha$ - and  $\beta$ -globulins in horse serum escaped detection owing to the limitation of the original Toepler schlieren method. Our separation of  $\alpha$ -globulin peaks reveals the double nature of  $\alpha$ -globulin in hog serum.

TABLE II

*Electrophoretic Analysis of Hog Serum and Various Fractions Obtained by Precipitation with Ammonium Sulfate*

The values reported in this table represent per cent protein and were computed from electrophoretic patterns by the usual method.

	$\gamma$ -Globulin	$\beta$ -Globulin	$\alpha$ -Globulin		Albumin
			$\alpha_1$	$\alpha_2$	
Hog serum.....	21.5	15.0	2.1	15.5	44.4
Activator.....	23.9	6.1	?	56.0	18.2
Fraction I ( $\gamma$ -globulin).....	95.0			5.1	
“ II.....	46.6	28.6	9.9	15.8	
“ III ( $\alpha$ -globulin).....	5.2			93.5	2.1
“ IV.....		8.5	5.9	54.4	27.5

Our quantitative estimation of whole serum by electrophoresis corresponded fairly closely to the values reported by Svensson, with the exception of albumin. In this investigation mobilities have been used only for the identification of components.

Preliminary tests indicated that the substrate for angiotonin formation is present in the activator fraction (1.22 to 2.10 M ammonium sulfate). A quantitative electrophoretic analysis of this fraction revealed the main constituent to be  $\alpha_2$ -globulin (Table II). Since the lower fraction on repeated precipitation up to 1.22 M ammonium sulfate turned out to be practically pure  $\gamma$ -globulin and since  $\gamma$ -globulin as well as albumin (Table I) was found to be devoid of physiological activity, it was concluded that  $\alpha_2$ -globulin is the substrate, since the content of  $\beta$ -globulin is very small. Although we were unable to prepare  $\beta$ -globulin in electrophoretically pure state,  $\alpha_2$ -globulin was obtained in a state of about 90 per cent purity (Fraction III). Fraction II (Table II) contains both  $\alpha_1$ - and  $\alpha_2$ -globulin

in addition to  $\beta$ - and  $\gamma$ -globulins. Angiotonin responses elicited by using this fraction as a substrate indicated that  $\alpha_1$ -like  $\gamma$ -globulin is inert. Since we can attribute a specific physiological function to the  $\alpha_2$ -globulin, it is unlikely that the existence of two  $\alpha$  components is due to individual variations of sera (9).

That  $\beta$ -globulin cannot act as substrate can be shown indirectly, for if it were effective much more angiotonin should be produced by incubation of Fraction II with renin than was actually observed. With  $\alpha$ -globulin as standard, all the angiotonin formed after incubation of Fraction II with renin could be accounted for if  $\alpha_2$ -globulin alone had reacted. If  $\beta$ -globulin were effective, the angiotonin responses should be at least 3 times higher than the values reported for Fraction II.

The fact that Fraction IV contains an unduly large amount of  $\alpha_2$ -globulin and only 30 per cent albumin indicates that the limit for the precipitation of albumin is very much higher in hog serum than in any other species. It may also explain the difficulties encountered in attempts to crystallize hog serum albumin. Precipitation at higher ammonium sulfate concentrations would probably yield a crystalline material. Although this fraction contains slightly more  $\alpha_2$ -globulin than the activator, physiological activity is entirely lacking (Table I). This can be explained by the presence of angiotonase, which destroys angiotonin just as soon as it is formed. In order to demonstrate the presence of this enzyme each fraction was tested by incubation with a known amount of angiotonin by a method previously reported (10). Only Fraction IV destroyed angiotonin, which indicated that angiotonase precipitates between 2.10 and 2.56 M ammonium sulfate. This fact has an important bearing upon the interpretation of the curves in Figs. 2 and 3. Assuming that the principle acting as substrate in the activator is  $\alpha_2$ -globulin, present to the extent of 56 per cent, its maximum amount of angiotonin produced after incubation with renin, expressed in terms of angiotonin units, should be much higher than the observed value. The albumin present in this fraction (18 per cent) probably carried with it a sufficient quantity of angiotonase to account for the difference. In a comparison of the maximum angiotonin rises of  $\alpha$ -globulin, activator, and whole serum at pH 6.5 in Fig. 3 the same phenomenon can be observed.

We have shown in a previous communication (4) that the reaction of renin and renin activator is enzymatic. Since it follows the laws of a first order reaction, the percentage of renin activator decomposed per unit of time must be proportional to the renin concentration. When the  $\alpha$ -globulin and activator curves in Fig. 2 are compared, it can be seen that the maximum amount of angiotonin which this protein can yield is reached at a lower renin concentration with activator than with  $\alpha$ -globulin. If the percentage of substrate decomposed per unit of time were the same,

the maximum value should be reached at the same renin concentration. This apparent discrepancy is due to the fact that in the  $\alpha$ -globulin experiments the specified amount of renin was added to 5 cc. of substrate and the solution made up to 7 cc., while in the activator experiment the final solutions were made up to 6 cc. The actual concentration of enzyme was therefore not the same in the two experiments. We did not feel justified in applying a correction factor in Fig. 2 because of the complexity of the system. However, if a correction 7/6 were made for the activator curve, the maximum angiotonin response would be reached at about 1.4 cc. of renin in both cases.

Various protein mixtures have been used as substrate for the preparation of angiotonin. Muñoz, Braun-Menendez, Faseiolo, and Leloir (3) and Page and Helmer (11) prepared a globulin fraction precipitated between 1.6 and 2.0 M potassium phosphate or 0.33 to 0.5 saturated ammonium sulfate. The latter fraction is about equivalent to the fraction precipitated between 1.25 and 2.10 M ammonium sulfate and may be assumed to have approximately the same composition as the fraction employed in this communication (activator). In a recent report by Sapirstein, Southard, and Ogden (12) on the use of renin activator for the restoration of blood pressure in hemorrhagic shock, a globulin fraction of ox blood was used. These authors followed the procedure of Braun-Menendez *et al.* (13) by precipitating serum proteins with 0.4 saturated ammonium sulfate. A comparison of the relative efficiency of serum, activator, and  $\alpha$ -globulin at various pH values is given in Fig. 3.

We have not analyzed the globulin fraction of Ogden and Braun-Menendez but the data of Svensson can be used to calculate the percentage of substrate in this fraction. When we use these data and neglect a small amount of euglobulin, the concentration of  $\alpha_2$ -globulin may be estimated as 26 per cent. Since the original hog serum contains approximately 14 per cent  $\alpha$ -globulin according to Svensson (9) and 15 per cent according to our analysis, the maximum amount of angiotonin which Ogden's activator could yield under the same conditions should be about twice the value of serum or one-half the value obtained with activator. If the favorable results obtained by Sapirstein, Southard, and Ogden (12) are due to arteriolar constriction (*in vivo* angiotonin formation), the use of the fraction obtained with 0.4 saturated ammonium sulfate would not be most efficient. Pure  $\alpha_2$ -globulin should give the best response per gm. of protein employed.

Other proteins such as hemoglobin, casein, egg albumin, etc., have been tested (3, 4) and found to be ineffective as substrates for renin. It is therefore not surprising that the action of this substrate is so specific that not even  $\alpha$ -,  $\beta$ -, or  $\gamma$ -globulin can replace it. A prosthetic group or peculiar arrangement of amino acids in the  $\alpha_2$ -globulin or some electrophoretically unresolvable fraction thereof must be responsible for this. The removal

of a prosthetic group by renin seems to us the most probable explanation, since the enzyme (renin) is not proteolytic in the conventional sense of the word.

We wish to acknowledge the valued assistance of Mr. Fredric R. Van Abeele in this study.

#### SUMMARY

An electrophoretic analysis of hog serum has been made which showed the presence of five distinct proteins.  $\alpha$ -Globulin was found to exhibit the phenomenon of a double peak. The serum was fractionated by precipitation with ammonium sulfate, which yielded some of the globulins in reasonably pure form.  $\alpha$ - and  $\gamma$ -globulins were found to precipitate within the concentration limits reported for horse serum, but the limits for albumin were found to be much higher.  $\beta$ -Globulin could not be obtained in pure state. Pseudoglobulin fractions were incubated with various amounts of renin and only the  $\alpha_2$ -globulin component found to act as substrate for the production of angiotonin. Euglobulins were entirely inactive. The relative efficiency of serum, activator, and  $\alpha$ -globulin as substrate was compared and the amount of angiotonin formed was found to be proportional to the concentration of  $\alpha_2$ -globulin. The substance referred to as renin activator is therefore identical to, or moves with the same electrophoretic mobility as  $\alpha_2$ -globulin.

An appreciable quantity of angiotonase has been demonstrated in the albumin fraction, while none could be found in the globulins.

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# PHOSPHOCREATINE AND INORGANIC PHOSPHATE IN WORKING AND RESTING MUSCLES OF RATS, STUDIED WITH RADIOACTIVE PHOSPHORUS

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The rapid hydrolysis of large quantities of phosphocreatine is one of the characteristic initial processes which occur during muscular contraction (1-3). Minimal concentrations are found after 1 or 2 minutes of work and little further change is apparent as work continues. The isotope technique would seem to offer a new approach to the question of whether the small quantity of phosphocreatine remaining after the beginning of work is involved actively in the continuation of this process. Sacks (4) measured the relative radioactivity of the phosphocreatine of cat muscle *in situ* after a 15 second period of isometric tetanus and found that the contraction did not modify the rate of turnover of the remaining phosphocreatine. We have now used radioactive phosphorus to study the effect of continuous isotonic contraction at the rate of three twitches per second on the phosphocreatine and inorganic phosphate of muscle, the effect of one period of work followed by rest, and the effect of repeated alternating periods of work and rest. The changes found in the concentrations of the phosphate compounds of the muscles studied under the conditions of work and rest in these experiments were similar to those previously reported (2). In muscle which had contracted 180 times in 1 minute, as compared with the resting control muscle of the unstimulated leg, these changes were found: approximately a 3-fold increase of inorganic phosphate, a decrease to a fifth of phosphocreatine, and a decrease to a half of adenosine triphosphate. As long as the muscle continued to work, no major changes in the content of these phosphates occurred. Subsequent rest of 5 minutes restored the resting values of inorganic phosphate and phosphocreatine, but the resynthesis of adenosine triphosphate was not as rapidly completed.

## Procedure

Sodium phosphate containing radiophosphorus was administered, generally intravenously, or in some of the longer experiments subcutaneously or intraperitoneally, to rats, from 30 minutes to 24 hours before the muscles were removed for analysis. Male rats, weighing about 240 gm., received from 1.6 to 7.5 mg. of phosphorus as dibasic sodium phosphate containing

from 39 to 188 microcuries of  $P^{32}$ . We are greatly indebted to Professor John H. Lawrence and Dr. Carl Helmholtz of the Radiation Laboratory, University of California, Berkeley, who have supplied the labeled sodium phosphate used in these studies.

The rats were anesthetized with sodium pentobarbital and the skin was loosened from the hind legs. The Achilles tendon of the left leg was loaded with a weight of 100 gm. and the muscle stimulated directly three times a second, each time with one complete cycle of 60 cycle current. The apparatus used for stimulation consisted essentially of a disk which rotates three times a second with a movable brush adjusted to make contact in a sector a twentieth of the circle, thus sending a complete cycle of 60 cycle current through the muscle three times each second with a voltage of 50 through a resistance of 40,000 ohms. The work done, as measured roughly with a Veeder counter, was greatest during the first 30 or 40 seconds, at the end of which time it decreased about a third and continued indefinitely at a constant rate. After varying intervals of work and rest the Achilles tendon was pulled upward and the flexor muscles of this leg were removed quickly and frozen in a mixture of carbon dioxide ice and alcohol. The muscles of the opposite control leg were then removed immediately in the same manner.

The frozen muscle was crushed between cooled steel blocks, quickly weighed, and dropped into tubes containing 20 ml. of ice-cold 5 per cent trichloroacetic acid and glass beads. The tubes were shaken in a room at  $0^{\circ}$  for 15 minutes and the solution was filtered. The acid-soluble phosphates were fractionated with barium hydroxide at  $0^{\circ}$  according to the modified Eggleton and Eggleton (5) technique (6); the barium-insoluble phosphates were dissolved with trichloroacetic acid and reprecipitated with barium hydroxide. The phosphate content was determined in duplicate by the method of Fiske and Subbarow (7).

Experiments in which sodium phosphate containing 0.5 and 0.7 microcurie of  $P^{32}$  was added to trichloroacetic acid extracts of four normal muscles prior to the foregoing fractionation showed contamination of the phosphocreatine with from 0.71 to 1.77 per cent of the inorganic phosphate. Such contamination would be serious, however, only when very different concentrations of phosphocreatine were to be compared, as in Table I. If, after the usual fractionation, inactive phosphoric acid was added to the barium-soluble fraction and this precipitated with barium hydroxide, the contamination of the phosphocreatine in four other muscles was reduced to 0.14 to 0.61 per cent of the inorganic phosphate. To minimize such contamination two additional precipitations of barium phosphate were introduced in the later experiments.

The radioactivity was measured with a scale-of-four, Geiger-Müller

counter of the immersion type (8). The phosphates of the muscle were first separated by the foregoing barium procedure and precipitated as ammonium phosphomolybdate at 0° overnight and then as magnesium ammonium phosphate for 4 hours at room temperature (9). Plasma inorganic phosphate was extracted with trichloroacetic acid and then precipitated directly as the magnesium salt. The magnesium ammonium phosphate was filtered, dissolved in hot 5 per cent nitric acid, and the radioactivity counted

TABLE I  
*Effect of Time on Distribution of Radioactive Phosphate in Inorganic Phosphate and Phosphocreatine of Working and Resting Muscle*

Time between injection of P <sup>32</sup> and taking muscle samples	Work	Inorganic phosphate			Phosphocreatine			
		Left	Right	Activity,* left	Left	Right	Activity*	
							Left	Right
min.	min.	mg. P per 100 gm.	mg. P per 100 gm.	per cent	mg. P per 100 gm.	mg. P per 100 gm.	per cent	per cent
60	1	60.4	24.8	42.4	7.2	53.0	18.4	14.7
60	30	77.2	25.2	43.7	4.2	53.3	25.4	19.4
60	30	69.7	28.6	55.2	8.4	54.4	40.5	29.7
60	30	63.6	18.8	39.3	9.4	54.4	22.0	16.1
60	30	69.5	21.2	31.9	8.4	47.8	13.8	10.5
61	30	59.2	20.0	43.6	9.0	46.3	18.8	15.8
63	1	68.0	26.2	38.8	6.6	52.4	30.6	19.2
63	45	65.0	18.5	29.6	13.9	56.3	11.3	9.1
75	60	50.2	25.7	68.0	18.8	50.0	35.2	19.8
120	1	72.0	25.3	56.5	7.4	48.4	69.0	40.3
125	120	54.6	30.2	72.0	9.9	38.6	65.7	27.5
132	60	74.0	32.2	64.5	7.4	40.0	46.9	28.8
180	30	67.5	23.8	81.6	6.8	46.2	95.9	69.9
180	30	60.3	19.6	57.4	4.2	50.0		
623	30	75.2	27.7	70.0	9.0	42.7	93.4	68.0
1320	60	45.4	28.5	95.5	24.5	42.3	89.5	87.2
1440	30	59.0	24.7	78.0	13.6	44.7	86.0	89.4

\* The radioactivity of the inorganic phosphate for each mg. of phosphorus of the resting muscle is considered as 100 and the radioactivity of all other fractions is expressed in percentage of this amount. In these experiments the left leg was stimulated for the indicated number of minutes immediately prior to taking the samples of muscles at the indicated times after administration of radioactive phosphate.

after suitable adjustment of volume. Differences of activity per mg. of phosphorus of nineteen samples of inorganic phosphate of muscle precipitated in duplicate varied from 1.0 to 4.5 per cent.

### Results

*Continuous Work*—Changes found in the concentrations of phosphocreatine and inorganic phosphate in muscles contracting 180 times a minute for

1 to 120 minutes were similar to those previously reported (2) (Table I). The concentration of the phosphorus of phosphocreatine in seventeen muscles which had worked in this manner averaged 9.9 mg. per 100 gm. in contrast to 48.3 in the opposite resting muscles; the concentration of the phosphorus of inorganic phosphate was 64.2 mg. in contrast to 24.8 mg.

The radioactivity per mg. of phosphorus of the inorganic phosphate and phosphocreatine of the resting muscles increased with time after the administration of the radiophosphorus. During the first 2 hours the radioactivity of the inorganic phosphate of the muscle varied from a twentieth to a sixth of that in the plasma, from the 3rd to the 10th hour from a twelfth to a half, and at 22 and 24 hours was approximately equal to that in the plasma. The uptake of radioactivity by the phosphocreatine was definitely slower than that of the inorganic phosphate of muscle. During the first 2 hours the radioactivity of the phosphocreatine was 9 to 40 per cent of the radioactivity of the inorganic phosphate of the resting muscle, from the 3rd to the 10th hour about 70 per cent, and at 22 and 24 hours about 90 per cent. All comparisons of radioactivity have been made on the basis of radioactivity for each mg. of phosphorus involved.

The radioactivities of the phosphate compounds in working muscles also showed an increase similar to that found in resting muscle with time after the administration of radiophosphorus. The actual Geiger-Müller counts of the phosphate fractions of muscle were from 10 to 1500 a second for each mg. of phosphorus, depending on the nature of the fraction, the time, and the amount of radiophosphate administered. For convenience of comparison the radioactivity of 1 mg. of the phosphorus of the inorganic phosphate of each resting muscle has been arbitrarily called 100, and the radioactivity of all other fractions expressed in percentage of this amount. The radioactivity of the inorganic phosphate per mg. of phosphorus in the working muscles was considerably lower than that in the resting muscles, particularly during the shorter periods after administration of radiophosphorus (when there is a marked differential distribution of radiophosphorus between the inorganic and organic phosphates of resting muscle). The decreased radioactivity of the inorganic phosphate of working muscle is undoubtedly due to dilution with phosphate liberated from the less radioactive organic compounds such as phosphocreatine and adenosine triphosphate. The fraction termed "inorganic phosphate" represents the fraction extractable with trichloroacetic acid which forms an insoluble barium salt and reacts directly with the Fiske and Subbarow reagent. Later in this paper data are presented which indicate that part of this fraction, especially in working muscle, is combined, probably with protein.

There was an increase of radioactivity of the residual phosphocreatine in some of the working muscles compared with their controls but this was

not progressive with the time of working. Moreover the possibility existed that this might be due to contamination of the very small quantity of phosphocreatine with traces of the highly active inorganic phosphate. Therefore two extra precipitations of barium phosphate from the phosphocreatine fraction were introduced in a later series of experiments (Table II). The radioactivities of the phosphocreatine in the muscles of the two hind legs of the control rats were found to be approximately the same. Likewise the radioactivity of the phosphocreatine of the muscles which worked 1 minute was similar to that of their resting controls. The radioactivity of the phosphocreatine in muscles which worked 60 minutes was not elevated greatly above the values in the resting leg. The increase found is extremely small compared with the radioactivity of the "inorganic phosphate" of the working muscle. If this were furnishing phosphate for any appreciable resynthesis during the 60 minute period of work, the radioactivity of the phosphocreatine should approach that of the inorganic phosphate.

The variability of the radioactivity between the muscles of the two hind legs of the control rats is probably due to inclusion of varying amounts of blood containing inorganic phosphate of high activity. This variation is not sufficient to alter the import of the values obtained. It thus appears that there is little if any increase of the radioactivity of the residual phosphocreatine in working muscles which can be attributed to the processes of work.

*One Period of Work Followed by Rest*—The muscles of the left hind legs of eight rats were stimulated to work for 3, 12, 48, or 55 minutes and then allowed to rest for 3, 5, or 12 minutes. The concentrations and radioactivities of phosphocreatine in the muscles which worked and rested were about the same as in the control resting legs, although approximately 80 per cent of the phosphocreatine in the working muscle would have been broken down during the 1st minute of work and resynthesized during the first minutes of rest (Table III). Thus the hydrolysis and resynthesis of 80 per cent of the phosphocreatine can be accomplished with no appreciable change of its radioactivity. Since the "inorganic phosphate" fraction of the muscle contained much greater radioactivity than the phosphocreatine, this must mean that the phosphate liberated by the hydrolysis of the phosphocreatine did not mix to any extent with the inorganic phosphate originally there and that resynthesis of phosphocreatine was accomplished, not from a mixed pool of "inorganic phosphates," but from phosphates of lower radioactivity similar to that of the phosphocreatine originally hydrolyzed.

The increased radioactivity of the inorganic phosphate found in some of the stimulated muscles might be due to the increased flow of blood produced by exercise, with the resultant inclusion of more of the highly active inor-

TABLE II

*Comparison of Radioactivity of Inorganic Phosphate of Plasma and Inorganic Phosphate and Phosphocreatine of Working and Resting Muscles*

Plasma inorganic phosphate activity	Muscle									
	Inorganic phosphate			Phosphocreatine						
	Left	Right	Activity, left	Left	Right	Activity				
						Left	Right	Left	Right	
Controls, no work; 90 min. between injection of $P^{32}$ and taking muscle samples										
per cent*	mg. P per 100 gm.	mg. P per 100 gm.	counts per mg. P	per cent*	mg. P per 100 gm.	mg. P per 100 gm.	counts per mg. P	counts per mg. P	per cent*	per cent*
649	34.9	33.4	497	94.5	50.8	42.5	158	175	30.0	33.3
1017	31.2	28.2	515	75.4	55.6	54.0	253	262	37.0	38.4
1555	35.4	32.2	508	89.4	48.8	51.5	123	132	22.0	23.6
1326	34.2	32.5	579	97.6	37.5	38.8				
2072	32.6	37.0	475	114	50.9	47.2	167	170	40.2	41.0
2117	25.3	31.4	231	124	51.1	60.6	57	53	30.5	28.3
1038	26.4	28.7	303	105	48.1	45.5	108	85	37.4	29.4

Left leg worked 1 min.; 90 min. between injection of  $P^{32}$  and taking samples†

930	63.4	33.2	281	67.5	7.9	48.4	160	148	38.5	35.6
810	61.2	34.1	403	56.0	20.3	50.2	390	374	54.2	51.9
1060	56.0	22.5	410	56.3	13.5	60.7	290	309	39.9	42.6
1525	54.4	28.4	415	64.4	26.3	54.2	159	175	24.7	27.2
1446	63.5	25.9	224	64.4	11.1	61.9	124	117	35.6	33.6

Left leg worked 60 min; 75 min. between injection of  $P^{32}$  and taking samples‡

756	41.6	28.3	642	79.2	17.0	39.4	213	164	26.3	20.2
1120	63.2	25.9	519	61.0	8.1	43.5	225	169	26.4	19.8
977	49.5	36.1	986	66.5	23.7	43.5	345	301	23.3	20.3
664	42.0	19.4	1434	62.9	14.0	50.0	305	239	13.4	10.5
596	66.3	24.0	435	33.2	11.0	48.1	178	129	13.6	9.8
732	71.0	26.0	489	35.2	11.4	57.7	252	118	19.4	9.1

\* The radioactivity of the inorganic phosphate for each mg. of phosphorus of the resting muscle is considered as 100 per cent.

† The left leg worked the 90th minute after injection of radioactive phosphate in the second group of experiments.

‡ In the third group the left leg worked from the 15th to the 75th minute after injection.

ganic phosphate of the plasma. The increase is surprisingly small compared with the great increase of the amount of blood flowing through working muscle.

*Repeated Short Periods of Work and Rest*—The muscles of the left hind

TABLE III  
Effect of One Period of Work Followed by Rest

Time between injection of $P^{32}$ and taking muscle samples	Work	Rest	Inorganic phosphate			Phosphocreatine			
			Left	Right	Activity,* left	Left	Right	Activity*	
								Left	Right
min.	min.	min.	mg. P per 100 gm.	mg. P per 100 gm.	per cent	mg. P per 100 gm.	mg. P per 100 gm.	per cent	per cent
30	3	12	22.4	21.9	92	50.3	52.5	6.8	7.4
112	3	12	42.5	19.0	51	33.9	47.6	13.9	9.4
116	3	12	30.6	29.0	120	45.5	46.9	20.6	18.2
60	12	3	22.7	29.7	118	53.9	54.2	20.6	20.7
126	48	12	39.4	30.2	113	43.7	49.8	37.5	32.3
1320	48	12	27.6	30.4	124	32.0	42.3	91.9	98.7
65	55	5	28.0	26.5	111	44.5	45.6	10.6	9.4
100	55	5	29.7	26.0	83	37.4	41.0	21.0	22.4

\* The radioactivity of the inorganic phosphate for each mg. of phosphorus of the resting muscle is considered as 100 per cent. The period of work of the left leg was performed so that the muscle at the end of the rest period was taken the indicated number of minutes after the injection of radioactive phosphate.

TABLE IV  
Effect of Repeated Short Periods of Work and Rest

Time between injection of $P^{32}$ and taking muscle samples	No. of repeated periods of work and rest; 1 min. work and 4 min. rest	Inorganic phosphate			Phosphocreatine			
		Left	Right	Activity,* left	Left	Right	Activity*	
							Left	Right
min.		mg. P per 100 gm.	mg. P per 100 gm.	per cent	mg. P per 100 gm.	mg. P per 100 gm.	per cent	per cent
60	8	27.8	24.3	88	44.7	58.7	14.3	11.4
90	9	34.9	26.4	78	42.0	51.8	38.4	41.4
90	10	26.4	29.1	123	46.8	46.7	33.0	30.0
180	6	28.9	27.0	90	47.5	43.2	49.6	42.8
180	6	24.0	28.2	115	48.8	48.8	80.6	79.5
275	6	26.6	31.6	105	42.5	39.1	52.8	67.7
623	6	23.8	26.9	104	45.2	40.7	74.9	72.5
1440	7	22.8	26.4	101	53.7	51.0	91.9	98.3
5 min. work and 5 min. rest								
60†	3½	83.6	43.0	56.5	8.1	26.9	20.4	18.4
75	6	26.5	26.4	104	48.6	48.2	20.1	20.6

\* The radioactivity of the inorganic phosphate per mg. of phosphorus of the resting muscle is considered as 100 per cent. The periods of work and rest of the left leg were performed the indicated number of times just prior to taking the samples of muscle at the indicated period after injection of radioactive phosphate.

† The rat died during the period of stimulation. The observed findings are typical for working muscle.



legs of eight rats were stimulated to work for 1 minute and then were allowed to rest for 4 minutes. This combination of work and rest was repeated six to ten times. In two additional rats combinations of 5 minutes of work followed by 5 minutes of rest were studied. Both the concentrations and activities of the phosphocreatine in the muscles which worked repeatedly were similar to those in the control resting muscles (Table IV). Thus even with repeated periods of work and rest, the repeated resynthesis of large amounts of phosphocreatine is accomplished from phosphates with radioactivity similar to that of the original phosphocreatine and adenosine triphosphate. None of the more radioactive "inorganic phosphate" originally present in the muscle is used in this resynthesis of phosphocreatine.

### *Comment*

The slowness with which the inorganic phosphate of muscle attains a radioactivity per mg. of phosphorus comparable to that of the plasma when  $P^{32}$  is administered has been demonstrated previously. Thus Hevesy (10) reported that after 4 hours of continuous administration of  $P^{32}$  to a rabbit the specific activity of the inorganic phosphate of the muscle was only 7.3 per cent of that of the plasma while the specific activities of the organic phosphates were even lower. An understanding of the slow penetration of inorganic phosphate into muscle would require some knowledge of the location of this in the muscle. Lipmann (11) stated that according to experiments of Eggleton (12) and Fenn (13) the inorganic phosphate is to be found in the extracellular fluid of resting muscle; Hahn, Hevesy, and Rebbe (14) stated that the amount inside the cells is 60 times larger than that in the extracellular fluid, while Furchgott and Shorr (15), who studied cardiac muscle, suggested that 30 to 40 per cent of the inorganic phosphate is extracellular.

The rate of penetration of labeled phosphate into the muscle was quite independent of the working process, in contrast to that of potassium, which has been studied by others. Thus Noonan, Fenn, and Haegge (16) found a 5-fold increase of the rate of penetration of radioactive potassium into rat muscle during 1 hour of stimulation and Hahn and Hevesy (17) found a 4-fold increase in the gastrocnemius of rats which swam for half an hour. An apparent decrease of radioactivity of the inorganic phosphate was found in the working muscles owing to dilution with phosphate hydrolyzed from the less radioactive organic compounds. As would be expected, the decrease found at the rate of work used in our experiments was greater than that found by Sacks (4) after a short period of tetanic stimulation.

Within the 1st minute of continuous work the phosphocreatine of the muscle is hydrolyzed so that less than 20 per cent of the amount found in resting muscle remains. This 20 per cent remains constant as long as work

is continued but is resynthesized to resting values again within 5 minutes of rest. If the working value is maintained by an equilibrium between hydrolysis and resynthesis, one might expect a more rapid incorporation of radioactive phosphocreatine during work. The radioactivity of the phosphocreatine increased with time after administration of radioactive phosphate but the increase was similar in resting and working muscle. The slight increase found with work after 60 minutes is probably no more than should be expected with a slow diffusion gradually reaching equilibrium at the altered level imposed by the working process. However, this gives no indication of the extent of hydrolysis and resynthesis of the phosphocreatine, except that if resynthesis did occur, it must have been accomplished directly from the same phosphate produced by hydrolysis.

That phosphocreatine is resynthesized from the products of its original hydrolysis is indicated by the fact that, after 80 per cent of the phosphocreatine has been hydrolyzed and resynthesized repeatedly by periods of work and rest, no greater radioactivity is found in the phosphocreatine of the exercised muscle than in the corresponding resting muscle. The amount of activity to be expected if the phosphate liberated from the phosphocreatine mixed with the inorganic phosphate and if the phosphate for resynthesis was taken from this mixed pool can be calculated roughly. For example in the first experiment in Table III there would have been about 40 mg. of phosphorus liberated from the phosphocreatine of 7.4 per cent activity and 10 mg. from the adenosine triphosphate of similar activity. If this were mixed with the 22 mg. of phosphorus of inorganic phosphate of 100 per cent activity, the pool of "inorganic phosphate" during the period of work would be 72 mg. with 35.6 per cent radioactivity. If subsequently 40 mg. were taken from this pool for resynthesis of the phosphocreatine, the latter should then have had an activity of 30 per cent instead of 6.8 per cent as found.

A study of adenosine triphosphate with radiophosphorus is in progress. The results indicate no marked increase in the conversion of labeled inorganic phosphate to adenosine triphosphate during work. With repeated periods of work and rest there is also no indication of increased radioactivity of the adenosine triphosphate.

An interesting hypothesis has been proposed in recent years postulating a direct reaction of the phosphate from adenosine triphosphate with myosin, by which phosphorylation energy is transmitted directly to the contractile (18-20) system. Engelhardt and Ljubimowa (21) first observed that myosin or a substance indistinguishable from it produces hydrolysis of adenosine triphosphate. Needham and others (22) then proposed a tentative hypothesis for the manner in which the cellular structures might function in a phosphate transfer system. Millikan (23) described this as

"the most promising attempt yet made to bridge the yawning chasm between metabolic processes and changes in structural organization accompanying activity." However, Kalekar (20) pointed out that no one has yet shown any direct evidence for such coupling of hydrolysis of phosphate with myosin. It would seem that our results with radiophosphorus may have some bearing on this. If phosphorylation of myosin is of major importance, phosphocreatine probably adds more phosphate, since it is hydrolyzed earlier and in greater amounts than is adenosine triphosphate. The amount of energy for each mg. of phosphorus involved is similar with the two compounds.

Our results indicate that the phosphate liberated by the hydrolysis of phosphocreatine and adenosine triphosphate as work begins does not mix to any appreciable extent with the labeled inorganic phosphate of the muscle and that this same phosphate (which has been split off from the creatine or other organic phosphates) is used for the rapid resynthesis of the phosphocreatine when rest ensues. This suggests the possibility that the liberated phosphate may be bound chemically or separated physically in the muscle from the inorganic phosphate originally there prior to extraction with trichloroacetic acid. That it might be bound merely because of its acidic nature is questionable, since lactic acid formed simultaneously from glycogen largely diffuses out of the muscle within 20 minutes. The phosphate might have some special function in relation to the contractile system, myosin, or it might be held to insure a rapid resynthesis of the phosphocreatine. In any case glycogen (24) is restored very slowly to a normal concentration when work is ended, whereas phosphocreatine is resynthesized within a few minutes.

#### SUMMARY

The penetration of labeled phosphate into muscle and its subsequent incorporation into the organic phosphates appear to go on at approximately the same rate in working as in resting muscles. If equilibrium, at the low level of phosphocreatine found in continuously working muscle, is maintained by continuous hydrolysis and resynthesis of phosphocreatine, resynthesis must be accomplished directly from the products of hydrolysis.

The increase of the inorganic phosphate, determined by extraction with trichloroacetic acid, of working muscle is due to the presence of phosphates released from the creatine, adenylic acid, and other organic phosphates by the processes of work. When the phosphates are labeled with radioactive phosphorus, it is evident that the phosphates released by hydrolysis of organic compounds do not mix to any appreciable extent with the more radioactive inorganic phosphate present in the muscle at the time of hydrolysis. When phosphocreatine is hydrolyzed and resynthesized during

periods of work and rest, its radioactivity is not increased by resynthesis from any of the originally present inorganic phosphate, but is the same as that of the phosphocreatine of the resting muscle. The same phosphate split from the organic phosphates is used for the resynthesis of phosphocreatine. It is possible that the phosphates hydrolyzed from the organic phosphates produce phosphorylation of myosin and that resynthesis is accompanied by dephosphorylation of myosin.

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# EFFECT OF SULFANILAMIDE ACCOMPANIED BY ACID OR ALKALI UPON THE ACID-BASE EQUILIBRIUM OF THE DOG. POSSIBLE INFLUENCE OF CARBONIC ANHYDRASE

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Soon after the introduction of sulfanilamide therapy, acidosis as an undesirable side reaction was reported. Southworth (1) observed clinical acidosis in two of 50 cases treated with the drug. In fifteen consecutive patients treated with sulfanilamide, all showed some decrease in  $\text{CO}_2$ -combining power of the plasma with an average decrease of 14.1 volumes per cent. A short time later Basman and Perley (2) noted similar decreases in the carbon dioxide content of the blood during sulfanilamide therapy. Mention was made that the urine was alkaline in such patients, although no data were given. Helmholtz (3) also reported at this time that the administration of sulfanilamide usually produces an alkaline urine. Marshall, Cutting, and Emerson (4) observed blood findings which were interpreted as acidosis in three dogs. In these dogs quantities of sulfanilamide much above the therapeutic level were administered and the changes in blood pH were not consistent. Hartmann, Perley, and Barnett (5) observed in two normal subjects and in two patients that the secretion of an alkaline urine and a decrease in serum  $\text{CO}_2$  content followed sulfanilamide administration. Inasmuch as the serum pH was slightly increased, the effect of sulfanilamide was interpreted as being an alkalosis resulting from primary hyperventilation.

The present report describes experiments which were carried out with dogs to determine more fully the effect of sulfanilamide on the acid base equilibrium. In these studies the effect of sulfanilamide alone was considered, as well as the effect of sulfanilamide when disturbance of the acid-base equilibrium was produced by the administration of sodium citrate or ammonium chloride.

## Methods

Adult female dogs were employed in all of the studies. The dogs were maintained on a diet of Purina dog biscuits. Blood samples were obtained from the jugular vein with minimum stasis and urine was obtained by catheterization. The blood was transferred under oil to special centrifuge

tubes and was centrifuged as soon as clotting occurred. The serum so obtained was employed for determination of  $\text{CO}_2$  content (6), pH (7), chloride (8), inorganic phosphorus (9), protein (10), and total base (11). A separate portion of blood was placed in a bottle containing dry potassium oxalate and this was used for sulfanilamide determination (12).

### EXPERIMENTAL

*Administration of Sulfanilamide*—The effects of a single dose of sulfanilamide on the acid-base equilibrium are shown in Table I. These results were obtained in five dogs which received by stomach tube 0.12 gm. of sulfanilamide per kilo of body weight. Samples of blood and urine were obtained during fasting, followed by blood samples at 1, 3,

TABLE I

#### *Blood Studies Following Single Dose of Sulfanilamide*

Average results obtained with five dogs are reported. 0.12 gm. of sulfanilamide was administered per kilo of body weight.

	Control	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
Blood sulfanilamide, mg. per 100 cc.....	0.0	13.0	11.7	9.9	7.5	3.2
Serum pH.....	7.38	7.38	7.38	7.37	7.38	7.38
“ total base, m.eq. per l.	156.0	151.3	153.0	154.2	154.4	154.6
“ chloride, m.eq. per l.....	112.7	108.9	110.4	109.9	109.9	110.3
“ bicarbonate, m.eq. per l.	23.0	21.9	21.4	21.4	22.4	22.5
Base combined with protein, m.eq. per l.....	15.0	13.9	14.3	13.8	14.2	13.2
Base combined with phosphate, m.eq. per l.....	3.1	2.9	3.0	3.3	3.1	3.6
Undetermined acid, m.eq. per l.	2.2	3.7	3.9	5.8	4.8	5.0
Urine pH.....	6.8	7.4	7.4	7.1	6.6	6.1

6, 12, and 24 hours and urine samples at 3, 6, 12, and 24 hours after administration of sulfanilamide. The levels of blood sulfanilamide obtained after this amount of drug simulate those sought in clinical practice in the treatment of severe infections (13). No appreciable changes in serum pH were observed. The serum total base decreased by approximately 5 milliequivalents per liter 1 hour after the sulfanilamide administration and then gradually returned toward the fasting level. Decreases in serum chloride and serum bicarbonate occurred, although these changes were quite small. Following the sulfanilamide the urine pH increased from 6.8 to 7.4.

*Administration of Sulfanilamide and Alkali*—In Table II the effect of administration of sodium citrate and sulfanilamide to normal fasting dogs

is presented. The plan of the experiment was similar to that described above. The quantity of sulfanilamide employed was the same (*i.e.* 0.12 gm. per kilo of body weight) and the quantity of sodium citrate used was 0.60 gm. per kilo of body weight. In Table III studies on two of the

TABLE II

*Blood Studies Following Single Dose of Sulfanilamide and Sodium Citrate*

Average results obtained with three dogs are reported. 0.12 gm. of sulfanilamide and 0.6 gm. of sodium citrate were administered per kilo of body weight.

	Control	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
Blood sulfanilamide, mg. per 100 cc.....	0.0	11.7	12.9	9.4	7.0	3.6
Serum pH.....	7.38	7.41	7.41	7.39	7.41	7.38
" total base, m.eq. per l....	161.4	159.3	159.8	159.5	158.6	160.4
" chloride, m.eq. per l....	112.3	111.6	109.1	106.2	110.2	112.3
" bicarbonate, m.eq. per l....	22.6	24.5	26.2	26.5	24.8	23.0
Base combined with protein, m.eq. per l.....	14.5	13.7	13.2	13.2	13.4	13.3
Base combined with phosphate, m.eq. per l.....	2.9	3.0	2.7	2.8	2.7	3.0
Undetermined acid, m.eq. per l....	9.1	6.5	8.6	10.8	7.5	8.8
Urine pH.....	6.8		7.8	8.1	8.0	6.5

TABLE III

*Blood Studies Following Single Dose of Sodium Citrate*

Average results obtained with two dogs are reported. 0.60 gm. of sodium citrate was administered per kilo of body weight.

	Control	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
Serum pH.....	7.41	7.52	7.54	7.50	7.47	7.42
" total base, m.eq. per l....	161.1	157.1	157.9	156.7	157.2	159.0
" chloride, m.eq. per l....	113.5	106.5	104.2	106.4	108.5	110.3
" bicarbonate, m.eq. per l....	26.2	29.6	30.9	30.3	28.7	26.6
Base combined with protein, m.eq. per l.....	14.1	13.8	13.5	12.8	12.5	12.5
Base combined with phosphate, m.eq. per l.....	2.2	2.1	2.3	2.3	2.1	2.4
Undetermined acid, m.eq. per l....	5.1	5.1	7.0	4.9	5.4	7.2
Urine pH.....	6.9		7.7	7.7	7.7	7.3

same dogs in which the same amount of sodium citrate alone was administered are shown. The level of blood sulfanilamide attained after sulfanilamide and sodium citrate was quite similar to that with sulfanilamide alone. The average serum pH after sodium citrate showed a maximum



increase of 0.13, whereas after sulfanilamide and sodium citrate the serum pH increased by only 0.03. The average increase in serum pH for the 1, 3, and 6 hour samples was 0.11 after sodium citrate and 0.02 after sodium citrate and sulfanilamide. The serum bicarbonate after sodium citrate showed a maximum elevation of 4.7 milliequivalents per liter and an elevation of 3.9 milliequivalents per liter after a combination of sulfanilamide and sodium citrate. After either sulfanilamide and sodium citrate or sodium citrate alone the urine pH was increased, with the increase being slightly greater after ingestion of a combination of sulfanilamide and sodium citrate.

*Administration of Sulfanilamide and Ammonium Chloride*—In Table IV the effect of administration of ammonium chloride and sulfanilamide

TABLE IV

*Blood Studies Following Single Dose of Sulfanilamide and Ammonium Chloride*

Average results obtained with three dogs are reported. 0.12 gm. of sulfanilamide and 0.37 gm. of ammonium chloride were administered per kilo of body weight.

	Control	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
Blood sulfanilamide, mg. per 100 cc.....	0.0	8.6	10.0	9.7	5.7	2.6
Serum pH.....	7.35	7.22	7.22	7.23	7.29	7.31
" total base, m.eq. per l....	151.3	149.3	149.4	148.7	149.3	151.6
" chloride, m.eq. per l. ....	108.3	113.7	116.3	116.2	114.7	113.9
" bicarbonate, m.eq. per l. ....	20.7	12.2	11.9	12.1	13.7	16.9
Base combined with protein, m.eq. per l.....	17.0	15.0	15.2	15.4	15.0	15.0
Base combined with phosphate, m.eq. per l.....	3.0	2.8	3.1	3.4	3.1	3.3
Undetermined acid, m.eq. per l. ....	5.3	5.6	2.9	1.6	2.8	5.5
Urine pH.....	5.4		5.1	5.2	5.4	5.5

to normal fasting dogs is presented. These experiments were similar to the preceding ones and involved the administration of the same amount of sulfanilamide. The quantity of ammonium chloride given was 0.37 gm. per kilo of body weight. On metabolism this amount of ammonium chloride yields a number of milliequivalents of acid that is equal to the number of milliequivalents of alkali that the sodium citrate yields on metabolism. In Table V are shown the results of administration of the same amount of ammonium chloride alone to the same three dogs. The level of blood sulfanilamide attained after dosage with sulfanilamide and ammonium chloride was slightly less than the blood level attained after dosage with sulfanilamide alone or with sulfanilamide and sodium citrate. The serum pH after sulfanilamide and ammonium chloride decreased by

0.13, whereas with ammonium chloride alone the maximum decrease was 0.09. The average decrease in serum pH for the 1, 3, and 6 hour samples amounts to 0.13 after ammonium chloride and sulfanilamide and is 0.07 after ammonium chloride alone. Serum bicarbonate fell by 8.8 milliequivalents per liter after sulfanilamide and ammonium chloride and 7.6 milliequivalents per liter after ammonium chloride alone. Urine pH after either ammonium chloride or ammonium chloride and sulfanilamide was rendered quite acid.

*Control Studies*—Since a considerable volume of blood was withdrawn in order to carry out the several analyses, two sham experiments were performed. In these experiments blood and urine were taken in the same manner as in the preceding studies and distilled water was given by stomach

TABLE V

*Blood Studies Following Single Dose of Ammonium Chloride*

Average results obtained with three dogs are reported. 0.37 gm. of ammonium chloride was administered per kilo of body weight.

	Control	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
Serum pH.....	7.35	7.26	7.28	7.29	7.32	7.33
" total base, m.eq. per l....	156.7	154.6	154.1	151.5	149.9	151.8
" chloride, m.eq. per l....	110.3	116.7	118.3	118.5	115.3	113.8
" bicarbonate, m.eq. per l.	22.2	15.3	15.0	14.6	15.8	17.9
Base combined with protein, m.eq. per l.....	15.7	14.9	15.0	14.6	14.6	14.5
Base combined with phosphate, m.eq. per l.....	3.1	2.9	3.2	3.4	2.9	3.0
Undetermined acid, m.eq. per l.	5.4	4.8	2.6	0.4	1.3	2.6
Urine pH.....	5.8		5.3	5.1	5.5	5.3

tube. The same analyses were made on the blood but no significant variation was observed in any of the constituents studied.

## DISCUSSION

The results of these experiments indicate that the effect of sulfanilamide on the acid-base equilibrium of the dog is not marked. These findings are in disagreement with the conclusions of Marshall, Cutting, and Emerson (4). However, the studies of Marshall and his associates involved dosage with amounts of the drug sufficient to cause marked dysfunction of the gastrointestinal tract and of the respiratory system.

A considerable amount of speculation has appeared regarding the effect of sulfanilamide on the acid-base balance. Mann and Keilin (14) found that sulfanilamide had a specific effect of inhibiting carbonic anhydrase,

the enzyme which plays an important rôle in carbon dioxide transfer. Since this observation of the effect of sulfanilamide on carbonic anhydrase, attempts have been made to prove or disprove the rôle of this enzyme in the changes in the acid-base balance of the body which occasionally result following sulfanilamide therapy (15). However, most of these attempts have proved unsuccessful and this can in part be explained by the fact that compensatory changes in respiration and renal activity tend to complicate the picture.

In the experiments in which sodium citrate was given alone, the serum pH increased by 0.13, whereas when sulfanilamide was given along with the sodium citrate the pH increased by only 0.03, although the rise in serum bicarbonate was similar in the two experiments. The average increase in pH for the 1, 3, and 6 hour samples was 0.11 after sodium citrate alone, whereas when sulfanilamide was given along with the sodium citrate the average increase in serum pH in the corresponding samples was only 0.02. When the method of Fisher (16) was used for the determination of "the significance of the difference of the means of small samples," the difference in pH change (after sodium citrate as compared with that after sodium citrate and sulfanilamide) was statistically significant, whereas the difference in bicarbonate change was not statistically significant. These findings lend support to the supposition that carbonic anhydrase is inactivated by sulfanilamide *in vivo*, since a loss of carbonic anhydrase activity would result in an increased accumulation of carbonic acid. This excess of carbonic acid prevents an increase in pH, even though the bicarbonate is increased.

In the experiments in which ammonium chloride was given alone the average decrease in serum pH in the 1, 3, and 6 hour samples was 0.07, whereas when sulfanilamide was given along with the same quantity of ammonium chloride the average decrease in serum pH in the corresponding samples amounted to 0.13. The average serum bicarbonate decrease in the corresponding samples was 8.8 milliequivalents per liter with ammonium chloride and sulfanilamide and 7.6 milliequivalents per liter after ammonium chloride alone. The difference in pH change was statistically significant by the method of Fisher (16) for the determination of "the significance of the difference of the means of small samples," whereas the difference in bicarbonate change was not statistically significant. The bicarbonate of the plasma is considered the first line of defense against acid, since acid will react with bicarbonate to form carbonic acid which dissociates as the blood passes through the lungs and gives rise to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This breakdown of carbonic acid to carbon dioxide and water is catalyzed by carbonic anhydrase. Therefore, if carbonic anhydrase activity is decreased by sulfanilamide, more carbonic acid would remain

in the blood, with a resultant decrease of serum pH. The fact that the serum pH is decreased more by ammonium chloride accompanied by sulfanilamide than by the same amount of ammonium chloride alone, whereas the serum bicarbonate changes are approximately the same, also suggests an *in vivo* inactivation of carbonic anhydrase by sulfanilamide.

Although both the acid and alkali experiments with sulfanilamide are consistent with an inactivation of carbonic anhydrase, these experiments do not conclusively prove the point, since primary alteration in pulmonary ventilation could have affected the changes observed.

#### SUMMARY

Studies of the acid-base equilibrium of normal dogs following administration of sulfanilamide are reported. Therapeutic quantities of this compound do not cause marked changes in the acid-base equilibrium in the dog.

Similar studies in which sodium citrate or ammonium chloride were given alone or along with sulfanilamide indicated that sulfanilamide prevented any increase in serum pH when given with alkali (sodium citrate) but that the decrease in serum pH after acid (ammonium chloride) was accentuated by sulfanilamide. These findings lend support to the inactivation of carbonic anhydrase by sulfanilamide *in vivo*.

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# FACTORS AFFECTING THE HYDROGEN ION CONCENTRATION OF THE CONTENTS OF THE SMALL INTESTINE\*

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That the intestine throughout its length adjusts the reaction of its contents to fit a definite pattern has been amply demonstrated in several species of animals (2-6). The present report deals with the acid-base alteration by which this adjustment is accomplished in dogs.

Average values for pH, bicarbonate, and  $\text{CO}_2$  tension in a number of loops in various sections of the intestines have been calculated from a rather extensive series of measurements. The changes during the adjustment of higher or lower initial values to those characteristic of the portion of the gut involved have been followed in several cases. Finally, the effects of ammonium chloride acidosis and sodium bicarbonate alkalosis on the final values were studied.

The experimental procedure and methods of analysis have been described in previous publications (7). Samples were collected under oil and the  $\text{CO}_2$  content determined by the manometric method of Van Slyke and Neill (8). The bicarbonate content and  $\text{CO}_2$  tension were calculated as outlined by McGee and Hastings (2).

As in previous investigations (7) acid and alkaline solutions of  $\text{CaCl}_2$ , hypo- and hypertonic with respect to blood, were used. They had the following approximate compositions: Solution I, hypotonic, acid, 150 milliequivalents of  $\text{CaCl}_2$  per liter, pH 4.5; Solution II, hypertonic, acid, 400 milliequivalents of  $\text{CaCl}_2$  per liter, pH 4.5; Solution III, hypotonic, alkaline, 150 milliequivalents of  $\text{CaCl}_2$  per liter, pH 7.3; Solution IV, hypertonic, alkaline, 400 milliequivalents of  $\text{CaCl}_2$  per liter, pH 7.3. They were circulated through the intestinal loops for 3 hours except in the experiments used to compile Fig. 1. Observations on human subjects were made by the Miller-Abbott intubation technique.

## Results

Table I shows the mean values of  $\text{pH}_{33}$ ,  $\text{pCO}_2$ , and  $\text{HCO}_3$  for five jejunal and four ileal loops in normal dogs. The sequence is the same as that in a

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this sequence is not known, since the exact location of a loop can be determined only on autopsy and several of these dogs are still in use. It can be stated, however, that they were in either the jejunum or ileum as indicated and that in Dogs 3 and 4 they were just above the ileocecal valve.

These means include values for all four solutions, since a statistical exam-

TABLE II  
*Composition of Blood of Dog 8*

Solution No.	Experiment No.	NH <sub>4</sub> Cl administered	Plasma CO <sub>2</sub> , acidotic dog			NaHCO <sub>3</sub> administered	Plasma CO <sub>2</sub> , alkalotic dog		
			Before experiment	Start of experiment	End of experiment		Before experiment	Start of experiment	End of experiment
		gm.	m.eq.	m.eq.	m.eq.	gm.	m.eq.	m.eq.	m.eq.
I	1	4	27.00	17.04	18.32	3	23.40	34.20	35.10
	2	4		17.38	19.48	3	21.60	34.20	31.50
	3	6	24.68	21.18	18.06	3		32.23	33.68
	4	6		13.70	12.43	3		35.08	37.20
	5	4		15.17	16.33	3		37.80	36.00
	6	4		16.40	17.83	3		40.47	38.40
II	1	4	25.20	14.65	16.14	2	24.73	35.10	33.29
	2	4		14.63	14.28	3	25.63	36.90	35.60
	3	4		15.90	16.63	3	27.30	45.05	30.15
	4	5		16.90	16.30	4	24.52	39.62	41.03
	5	5	27.02	18.45	17.52	4	20.25	28.37	23.70
	6	3		13.18	12.82	15	22.50	32.08	32.52
III	1	6		12.43	12.03	15			
	2	5		14.24	14.53	15	23.86	38.90	37.72
	3	6	27.52	17.97	16.58	15	24.73	39.70	40.16
	4	5		18.92	19.41	15	27.43	40.20	38.70
	5	6		17.30	17.54	15		36.03	29.36
	6	6		18.00	17.76	15			
IV	1	7		12.31	13.87	16		38.70	40.93
	2	6		14.17	13.23	15	25.98	36.50	39.30
	3	6		15.12	14.54	15			
	4	9		12.68	13.23	18			
	5	7		15.03	15.62	17			
	6	6		15.07	14.85	17			

ination showed that in normal animals there is no significant difference between such values.

Berk, Thomas, and Rehfuess (1) found that the over-all pH of the stomach of fasting dogs was less than that of the stomachs of fasting humans but that the reactions of the duodenal bulbs were the same. Apparently the similarity continues into the jejunum. Our pH measurements on dogs agree with those of McGee and Hastings and our own on humans. These last authors studied pure jejunal juice, while we used solutions and our



TABLE III  
Final Compositions of  $\text{CaCl}_2$  Solutions in Dog 8

Solution	$\text{pH}_N^\circ$			$\text{HCO}_3$			$\text{pCO}_2$		
	Untreated	Acidotic	Alkalotic	Untreated	Acidotic	Alkalotic	Untreated	Acidotic	Alkalotic
I. Hypotonic, acid	7.06	6.94	7.16	5.48	3.55	6.63	20.5	17.2	19.5
	7.10	6.96	7.20	2.53	3.35	7.63	11.1	16.1	20.3
	6.75	7.14	7.10	2.27	4.51	6.60	17.4	13.8	22.1
	6.75	7.08	7.16	4.14	4.08	8.08	31.6	14.0	23.6
	6.86	7.02	7.06	4.50	5.58	6.63	26.7	22.5	24.5
	6.86	6.88	7.32	5.35	3.78	9.83	31.8	21.0	19.1
Mean.....	6.90	7.00	7.16	4.05	4.14	7.53	23.2	17.4	21.5
P.E.M.....	$\pm 0.0425$	$\pm 0.0264$	$\pm 0.0247$	$\pm 0.3878$	$\pm 0.2240$	$\pm 0.3970$	$\pm 2.2820$	$\pm 1.0330$	$\pm 0.6320$
II. Hypertonic, acid	7.26	6.83	6.83	6.54	3.18	5.91	14.9	17.3	33.0
	7.06	6.83	6.72	7.20	3.56	4.54	24.0	20.0	32.5
	6.96	6.85	6.73	3.63	4.07	5.54	15.3	22.1	38.9
	6.86	6.80	6.82	2.89	3.61	7.02	15.6	21.5	39.5
	6.85	6.85	6.96	2.70	3.62	7.07	14.3	19.2	29.8
	6.96	6.84	6.96	5.03	3.69	5.79	21.0	20.1	24.4
Mean.....	6.99	6.83	6.84	4.67	3.62	5.98	17.5	20.0	33.0
P.E.M.....	$\pm 0.0422$	$\pm 0.0240$	$\pm 0.0247$	$\pm 0.2624$	$\pm 0.0767$	$\pm 0.2630$	$\pm 1.1025$	$\pm 0.4710$	$\pm 2.0050$

III. Hypotonic, alkaline	7.06	6.87	7.27	4.63	5.04	8.83	18.8	28.7	19.3
	6.95	6.92	7.19	5.01	4.93	10.34	25.2	25.0	27.3
	6.85	6.94	7.24	4.40	3.56	8.55	25.4	17.3	20.5
	6.76	7.03	7.37	4.35	6.44	9.85	31.9	25.4	17.5
		6.83	7.28		3.60	7.37		22.5	18.1
		7.13	7.04		8.63	7.70		26.4	29.4
Mean.....	6.90	6.95	7.23	4.61	5.37	8.77	25.6	24.2	22.0
F.E.M.....	$\pm 0.0450$	$\pm 0.0822$	$\pm 0.0150$	$\pm 0.0303$	$\pm 0.5280$	$\pm 0.3220$	$\pm 1.8140$	$\pm 1.0770$	$\pm 0.6220$
IV. Hypertonic, alkaline	6.85	6.98	7.24	3.42	5.41	8.77	18.9	22.2	18.5
	6.96	6.88	7.20	5.29	4.97	7.07	22.5	25.1	16.2
	6.86	6.87	7.25	3.76	4.76	9.66	32.5	24.5	20.7
	7.05	6.92		4.75	4.13		16.5	19.1	
		6.78			3.64			23.1	
		6.85			4.28			23.1	
Mean.....	6.93	6.88	7.23	4.31	4.53	8.50	22.6	22.8	18.5
F.E.M.....	$\pm 0.0315$	$\pm 0.0478$	$\pm 0.0000$	$\pm 0.3352$	$\pm 0.1755$	$\pm 0.0515$	$\pm 1.5340$	$\pm 0.7030$	$\pm 0.8035$

results reflect the effect of dilution. Hence, our figures for  $\text{CO}_2$  tension and  $\text{HCO}_3$  in dogs and humans are lower than theirs. But our values for dogs are of the same magnitude as those for our human subjects.

The figures seem to indicate that the increase in pH in the jejunum is due to combined decrease in  $\text{CO}_2$  tension and increase in bicarbonate. In the ileum the picture abruptly changes and the ileal loops, with the exception of the last one, show a relatively high  $\text{HCO}_3$  with  $p\text{CO}_2$  values of average magnitude.

McGee and Hastings have made the suggestion that there are cells in the intestine that can secrete acid and others that can secrete base. If this is correct, it appears from our figures that the high pH in the ileum is due to an increased secretion of base.

Fig. 1 illustrates the progress of adjustment of acid and alkaline solutions to the composition characteristic of the individual loops.

As has been pointed out previously (7), the pH of alkaline solutions introduced into loops is promptly reduced to acid levels and then is raised to the final value. The initial step takes place so rapidly that the acid-base changes are difficult to follow. Fig. 1 shows that in the changes subsequent to this initial phase there is no consistent difference between solutions acid and alkaline to start with. In two experiments with alkaline  $\text{CaCl}_2$  solutions, Experiments 3272 and 794,<sup>1</sup> and in an experiment with normal  $\text{NaCl}$  solution (not shown in Fig. 1) the pH values were going down when the first observations were made. The graphs reveal a pronounced rise in  $p\text{CO}_2$  during this first phase. The subsequent rise in pH is accomplished by a rise in  $\text{HCO}_3$ .

Two explanations are offered for the sharp decrease in the pH of alkaline solutions to values below the equilibrium ones: (1) gaseous  $\text{CO}_2$  may diffuse more rapidly through the intestinal wall than does bicarbonate, thus causing a temporary accumulation which is later neutralized as base comes in; (2) if the suggestion of McGee and Hastings is correct, the relative amounts of acid and base secreted by the intestine may cause temporary alterations in the pH.

Table II gives the data on the composition of blood of Dog 8 which was made acidotic with  $\text{NH}_4\text{Cl}$  and alkalotic with  $\text{NaHCO}_3$ . The changes in composition of solutions under this treatment were compared with those in the untreated animals in Table III. A statistical analysis of these figures shows that in the untreated animal there is no significant difference in the final pH of a solution because of its initial concentration or pH. Any attendant differences in  $\text{HCO}_3$  or  $p\text{CO}_2$  are due to chance. The same ap-

<sup>1</sup> The  $\text{CO}_2$  determination for the 30 minute sample was lost; so  $p\text{CO}_2$  and  $\text{HCO}_3$  could not be calculated. The pH value for this sample was 5.8, the original solution having a pH of 7.2.

pears to be true of the acidotic state. On the other hand, alkalosis causes significant increases in the final pH in comparison with the normal and acidotic states of all solutions except the hypertonic acid ones. The high pH values were due to increased  $\text{HCO}_3$ , the tension remaining within the normal range. In the case of the hypertonic acid solutions the increased  $\text{HCO}_3$  was counterbalanced by a large increase in  $\text{CO}_2$  tension which resulted in a normal pH. McGee and Hastings observed that human jejunal juice maintains a constant tension when  $\text{HCl}$  or  $\text{NaHCO}_3$  is introduced into the gut. A similar situation seems to exist in intestinal contents (except as noted) when the blood itself is made acidotic or alkalotic.

The observation that ammonium chloride acidosis produces no change in the reaction of intestinal contents is of interest because of its supposed aid in the absorption of calcium. If it has such an effect, it obviously is not because it makes the intestine more acid.

#### SUMMARY

Studies of the acid-base conditions in intestinal contents indicate that the progressive increase in pH throughout the jejunum is due to a combined increase in bicarbonate and decrease in  $\text{CO}_2$  tension. The high values in the ileum are due to increased bicarbonate.

The sharp drop in pH of alkaline solutions introduced into the intestine is due to a relatively rapid influx of  $\text{CO}_2$ . Subsequent formation of bicarbonate returns the values to the equilibrium point.

Ammonium chloride acidosis causes no significant alteration in the reaction of intestinal contents, and hence does not increase calcium absorption by increasing the acidity of the solutions in the gut. Bicarbonate alkalosis, on the other hand, produces a rise in pH.

The reaction of the human jejunum is the same as that of the dog.

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# A GROWTH STIMULANT FOR *LACTOBACILLUS CASEI*

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In studies with *Lactobacillus casei*, it was observed that a striking stimulation in growth resulted when natural extracts were added to a medium which contained all the known essential growth factors for this organism. This effect was observed in short term growth tests (14 to 16 hours), and practically disappeared in a 3 day test. There was, apparently, an unknown substance or substances capable of stimulating the growth of *Lactobacillus casei* in the early stages. Since it has become necessary for us to discontinue this project indefinitely, the preliminary work on the nature of the stimulant is reported at this time.

**Medium**—1 liter of the basal medium contained 200  $\gamma$  each of calcium pantothenate, riboflavin, nicotinic acid, pyridoxine, and thiamine, 0.5  $\gamma$  of biotin, 200 mg. units of folic acid, 5 mg. of inositol, 20 mg. each of adenine sulfate, guanine hydrochloride, uracil, and xanthine, 10 gm. of Difco technical Casamino acids, 200 mg. each of tryptophane and cystine, 12 gm. of sodium acetate, 20 gm. of glucose, 1 gm. each of potassium mono- and dihydrogen phosphates, 0.4 gm. of magnesium sulfate heptahydrate, and 0.02 gm. each of sodium chloride, ferrous sulfate heptahydrate, and manganese sulfate monohydrate. The pH was adjusted to 6.7 before use.

## Assay Method

Aqueous solutions to be tested for growth activity were placed in 2  $\times$  10 cm. test-tubes, and the volumes made up to 2.5 cc. with distilled water. To each of these tubes were then added 2.5 cc. of the medium. The tubes were plugged with cotton, autoclaved at 15 pounds pressure for 15 minutes, and cooled. To each of the tubes was then added 1 drop of a light saline suspension of *Lactobacillus casei*, which had previously been grown for 24 hours in the inoculum of Pennington, Snell, and Williams (1). The inoculated tubes were incubated in an oven at 37° for 14 to 16 hours and the extent of growth was determined quantitatively by measuring the turbidities of the resulting cultures in the Williams thermoelectric turbidimeter (2). It is probable that the production of lactic acid could also be used. In 15 to 21 hours, the addition of 400  $\gamma$  of peptone led to the

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production of almost twice as much acid as there was in the blanks. In 69 hours, there was no difference in this respect between the tubes containing peptone and the blanks.

### Results

Many plant and animal tissue extracts were found to produce increased growth over the blanks in this test, although considerable variation in relative effect was observed. In the work reported here, Wilson's bacteriologic peptone was used as the source of the growth factor. The effect of graded additions of this peptone on the growth of the test organism is

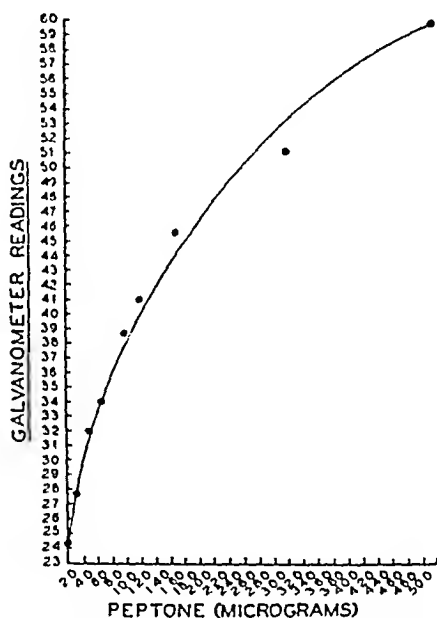


FIG. 1. Effect of graded additions of peptone on the growth of the test organism

shown in Fig. 1. All test solutions were compared with a peptone "standard" which was always run at the same time.

To determine whether the effect could be due to deficiencies of known materials, a number of tests were run. Doubling and tripling the content of the B vitamins led to no increase in growth over that in the blanks, indicating the sufficiency of the basal medium in this respect.

The following compounds were found to be without effect when tested individually: glycine, *l*-alanine, *l*-leucine, *dl*-isoleucine, *l*-aspartic acid, *l*-glutamic acid, *dl*-valine, *l*-histidine, *l*-tryptophane, *l*-proline, *l*-hydroxyproline, *dl*-serine, *dl*-threonine, *l*-tyrosine, *l*-cystine, *dl*-methionine, *dl*- $\beta$ -

phenylalanine, *L*-lysine, *L*-arginine, *L*-asparagine, ornithine, *dl*- $\alpha$ -amino-*n*-butyric acid,  $\alpha$ -aminoisobutyric acid,  $\beta$ -amino-*n*-butyric acid, *dl*- $\alpha$ -amino-*n*-caproic acid, hydroxylysine,  $\epsilon$ -amino-*n*-caproic acid, *dl*- $\alpha$ -aminocaprylic acid, *dl*- $\alpha$ -amino- $\alpha$ -methylbutyric acid, *dl*- $\alpha$ -amino-*n*-butyric acid, *dl*- $\delta$ -amino-*n*-valeric acid, *d*-glucosamine, glutathione, creatine, creatinine, acetylcholine, choline, ethanolamine, pimelic acid, glycolic acid, raffinose, arabinose, melibiose, galactose, ascorbic acid, fucose, maltose, mannitol, mannose, pyrazinemonocarboxylic acid, pyrazinedicarboxylic acid, *p*-aminobenzoic acid, thiochrome, indolylbutyric acid.

A mixture of the first twenty amino acids produced a slight stimulation in growth, equivalent to approximately 5 per cent of the response exhibited by a like quantity of peptone.

Of all the pure chemicals tested, glutamine was the only one to give a strong growth response. It was about 5 to 10 times as potent on an equal weight basis as Wilson's peptone, which would indicate that, if glutamine were the active ingredient, it must comprise 10 to 20 per cent of the latter. However, glutamine was much more readily hydrolyzed than the peptone factor, and Feeney and Strong (3) have shown that glutamine produces a strong growth effect in a medium containing a large amount of alkali-treated peptone. We have found that mild alkali treatment (as in the preparation of the peptone for the Feeney-Strong medium) does not destroy the peptone factor, and, since glutamine has thus been shown to produce a strong effect which the peptone factor cannot duplicate, the non-identity of the two is fairly well established. To confirm this point, the work of Feeney and Strong was repeated and extended. A medium similar to theirs was constructed, with Wilson's peptone. In the presence of excess pantothenic acid, glutamine gave a strong growth response over the blanks in a 14 hour growth test, whereas the addition of peptone was without effect.

It is probable further that the peptone factor is not a peptide of glutamine, for a sample of peptone which was hydrolyzed in the presence of a mixture of proteolytic enzymes (caroid, clarase, trypsin, and polidase) also failed to give the glutamine growth response in the Feeney-Strong test. This indicates that no free glutamine was formed by enzymatic hydrolysis of the peptone, although the possibility remains that glutamine may have been formed and been hydrolyzed further to glutamic acid, which would be inactive in this test.

Other investigators have obtained evidence for additional factors in natural extracts stimulating the growth of *Lactobacillus casei* (4-8), but, since these effects were all observed in basal media containing considerable amounts of peptone, it is evident that the responsible stimulants are different from the peptone factor described here.



*Nature of Growth Factor*—The factor appears to be fairly stable in neutral or weakly acidic or alkaline solutions. No destruction occurred on heating at 100° for 2 hours within a pH range from about 2 to 11. At pH 12 or higher under these conditions, a 50 per cent loss in activity occurred, which may indicate racemization. Strong acid is very destructive, most of the activity disappearing when the peptone was heated in 0.7 N or stronger sulfuric acid solution. Attempts to esterify the factor by heating in alcoholic hydrogen chloride or sulfuric acid were without avail, owing to the extensive destruction which occurred.

Extraction of the solid peptone with methanol, ethanol, acetic acid, pyridine, aniline, and mixtures of these with water did not yield any extracts which were more potent than the original peptone.

Studies of the tendency of the factor to migrate towards the electrodes in an electrolytic arrangement (9) indicated that it contained both acidic and basic groups. The greatest concentrations were found in the cups which were in the pH range from 3.5 to 4.5, indicating an isoelectric point in that region.

It is probable that the factor does not contain any peptide linkages, for no increase or loss in activity was observed on hydrolyzing a sample of peptone in the presence of a mixture of proteolytic enzymes (caroid, clarase, trypsin, and polidase).

One of the outstanding characteristics of the factor was its resistance to adsorption. No, or very little, selective adsorption was observed when aqueous solutions of peptone were shaken with 25 per cent of the solids weight of activated alumina, Darco G-60 charcoal, silica gel, titania, bentonite, or montmorillonite. Varying the pH from 3.0 to 8.0 or heating at 100° or both had only small effects. The adsorption on charcoal was not appreciably greater when methanol was used as the solvent. By using large amounts of charcoal (300 per cent of the solids weight), approximately 70 per cent of the activity could be removed from an aqueous solution at its natural pH, but 60 per cent of the total solids was also removed, thus again showing only slight differential adsorption. Elution of this charcoal adsorbate has not yet been studied, and it is possible that preferential elution may be effected.

Partial precipitation of the activity was observed when aqueous solutions were treated with various heavy metal salts. Mercury and silver in alkaline media appeared to be the most effective precipitants. The factor was not precipitated by phosphotungstic acid, picric acid, or nucleic acid. Flavianic acid was more effective, but the precipitation was not selective, and the potency of the precipitated matter was not higher than that of the solids in the filtrate.

On the assumption that the factor might contain a carboxylic acid

function, the procedure for the preparation of the brucine salt was followed, and the dry solid extracted with chloroform and ethanol. Only small amounts of activity were extracted and no concentration was effected.

#### SUMMARY

1. The presence in natural extracts of a substance of unknown nature which stimulates the early growth of *Lactobacillus casei* is demonstrated.

2. The properties of the growth factor indicate it to be very water-soluble, and not very soluble in organic solvents. It is stable in weakly acidic or alkaline solutions, but is destroyed by strong acid or alkali. It is probably not a peptide and appears to be amphoteric, with an isoelectric point in the pH region from 3.5 to 4.5. It is very resistant to adsorption and is partially precipitated by flavianic acid and heavy metals.

We wish to thank Miss Jean Taylor for her assistance.

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# THE ABSORPTION AND RETENTION BY DOGS OF SINGLE MASSIVE DOSES OF VARIOUS FORMS OF VITAMIN D\*

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In a previous study (1), it was found that large amounts of vitamin D as delsterol (presumably vitamin D<sub>3</sub>) caused greater damage in rats fed varying amounts of Ca, P, and vitamin A than did corresponding excessive amounts of vitamin D as irradiated ergosterol or calciferol (vitamin D<sub>2</sub>). However, the rats later relieved of excess delsterol recovered growth more rapidly and presented better condition in both the soft tissues and the bones than did the rats relieved of the corresponding excess of vitamin D<sub>2</sub>. These notes on the progress of, and the recovery from, vitamin D hypervitaminosis suggested the view that irradiated animal sterol, vitamin D<sub>3</sub> as delsterol, may be more rapidly and completely absorbed than the irradiated plant ergosterol or calciferol, vitamin D<sub>2</sub>, and is likewise more rapidly and completely excreted. The present study was undertaken to determine whether such differences in absorption, excretion, and retention could be detected when vitamin D from various sources was given. One moderately large dose of vitamin D in the form of irradiated ergosterol, activated cholesterol (delsterol), or tuna liver oil was given orally to dogs and the presence of the vitamin was followed in the blood and the feces as well as its effect on serum calcium for 180 days. These dogs were later given a single massive dose, 200,000 U.S.P. units per kilo of body weight of irradiated ergosterol and delsterol respectively, and after 3 days were sacrificed and their tissues examined for vitamin D content.

## EXPERIMENTAL

Three pure bred cocker spaniel dogs of the same litter, born in the laboratory colony, were weaned when 1 month old and placed on the following diet: granulated sugar 20.9, corn-starch 8.4, raw casein 45.8, fat 10.0, Salts 5 (2) 2.4, CaCO<sub>3</sub> 1.5, wheat germ 5.0, brewers' yeast 6.0 per cent. This diet, which is the stock diet for the colony, provided approximately 40 per cent protein, 0.96 per cent Ca, and 0.74 per cent P and, supplemented by the fat-soluble vitamins, was apparently adequate in all respects for the growth of these animals.

The dogs were kept in individual metabolism cages in the laboratory

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during the entire period of their lives, 12 to 14 months. They were never exposed to direct sunlight.

At first 50 gm. of dry food daily per kilo of body weight were fed and the amount decreased with diminishing needs and appetite of the animals to 30 gm. after 4 months and to 25 gm. per kilo after 7 months. During the 1st month after they were weaned the dogs received 1 gm. of cod liver oil per kilo of body weight per day and sufficient shark liver oil to supply an additional 800 units of vitamin A per kilo per day. The cod liver oil was removed when the dogs were approximately 2 months old, and the vitamin A continued in the form of diluted shark liver oil at the same level of 800 units per kilo per day. This amount of shark liver oil supplied negligible amounts of vitamin D (0.05 u.s.p. unit per kilo per day). After 2 months of this depletion when no detectable amounts of vitamin D appeared in the feces, one dose of vitamin D was given in the following forms, each of which had been reassayed to establish its vitamin potency.

*Tuna Liver Oil (Katsuwonus pelamis)*<sup>1</sup>—The original preparation which contained 120,000 u.s.p. units of vitamin D and 40,000 units of vitamin A per gm. was diluted with an equal weight of corn oil. This was given to Dog 330, a female.

*Irradiated Ergosterol*<sup>1</sup>—The original product which contained 1,000,000 u.s.p. units per gm. was dissolved in corn oil and sufficient shark liver oil was added to produce the same concentrations of vitamins A and D which were present in the diluted tuna liver oil. This was given to Dog 331, a female.

*Activated Cholesterol (Delsterol)*<sup>2</sup>—The original product which contained 300,000 u.s.p. units of vitamin D was dissolved in corn oil and sufficient shark liver oil was added to give the same concentrations of vitamins A and D as were in the diluted tuna liver oil. This was given to Dog 328, a male.

Each animal received orally in a gelatin capsule on June 13, 1940, when it was 117 days old, one dose of the prescribed oil sufficient to provide 20,000 units of vitamin D and 6667 units of vitamin A per kilo of body weight.

Blood samples taken before and at intervals after the dosing were examined for serum calcium and vitamin D content. Feces were collected daily for the first 4 days, then pooled into weekly collections for vitamin D assay.

<sup>1</sup> We acknowledge with gratitude the gift of the tuna liver oil and of the irradiated ergosterol from Mead Johnson and Company, Evansville, Indiana, through C. E. Bills.

<sup>2</sup> We are indebted for this product to J. Waddell of E. I. du Pont de Nemours and Company, Inc., New Brunswick, New Jersey.

### *Methods*

The method used for the vitamin D assay of the feces was essentially that of Heymann (3). The feces were dried at 80°, weighed, covered with 20 per cent KOH in ethyl alcohol, and heated on a water bath for about 3 hours. The still hot, but liquid material was extracted five times with 300 to 600 ml. portions of ethyl ether, the combined ether extract washed with water until the washings became colorless, dried with anhydrous  $\text{Na}_2\text{SO}_4$ , and the ether distilled off. The residue was dissolved in corn oil in an amount equal to one-sixth of the original dry weight of the feces. The extract was administered by pipette for 8 consecutive days to standard rachitic animals. The rats were sacrificed after the 10th day, the tibias were removed, cleaned, split, and stained with silver nitrate, and the line test readings made.

Blood samples were taken from the femoral vein, centrifuged, and the serum separated and kept frozen until it was used for assay. The serum was injected intramuscularly into standard rachitic rats daily for 8 days, a total of 3 ml. of serum being administered, and the rats were killed after 10 days. The tibias were removed for line test readings as above.

Calcium of the serum was determined according to the method of Larson and Greenberg (4) and inorganic phosphate by that of Fiske and Subbarow (5).

### *Results*

The single moderate dose of 20,000 units of vitamin D per kilo of body weight produced no visible deleterious effect on growth, appetite, or the general behavior of the dogs (Fig. 1). Radiographs of the wrists taken at intervals during the 6 months following the dosing indicated that no rickets developed. This strain of cocker spaniels has been found (6) to require about 72 units of vitamin D per kilo per day during the first 4 or 5 months after weaning in order to insure normal bone development. It may be that young dogs, even more appropriately than human infants, can be economically and successfully protected against rickets by the vitamin D-Stoss treatment.

The results of the vitamin D assay of the blood and feces and of the serum calcium determinations are noted in Table I. In no case was an appreciable amount of vitamin D excreted after the 1st day. This is in marked contrast with the results of Heymann (7) who found vitamin D persistent in the feces of two adult dogs for 8 months after the administration of viosterol and for at least 6 months after the administration of drisdol. The vitamin D was given in somewhat larger amount than was used in the present study. Explanation for the differences may be found in differences in the degree of previous vitamin D depletion of the animals

and in differences in their ages. The growing puppies which were used in this experiment may have utilized or stored vitamin D more quickly or efficiently to meet growth requirements than did the adult dogs of Heymann's experiment. Windorfer (8) who gave single massive doses to rachitic infants also found that there was practically no excretion of vitamin D in the feces after the first few days.

No evidence of reexcretion of vitamin D through the gut was obtained during the 26 days of continual collection of feces following the dosing nor in the 4 weeks collection made during the 3rd month thereafter.

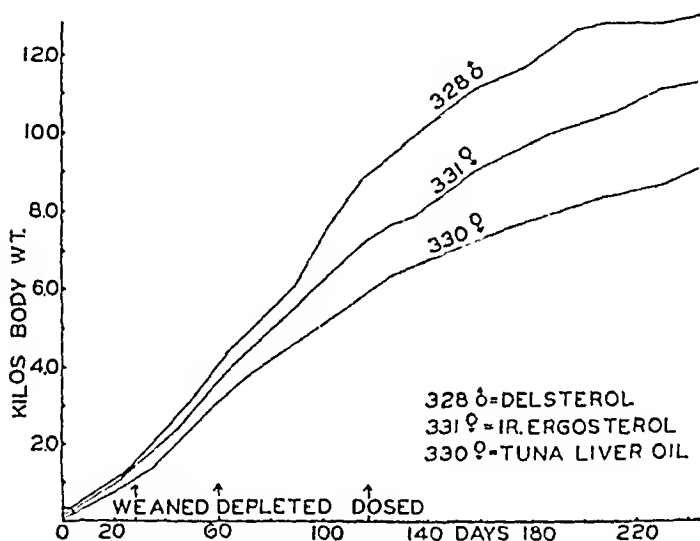


FIG. 1. The growth of three cocker spaniel dogs from birth to 8 months of age, as affected by administration of one dose of vitamin D, 20,000 u.s.p. units per kilo, when 117 days old.

In order to exclude the possibility of differences in the capacity of the dogs to absorb fat, balance studies were made by the method of Fowweather (9) for determination of total fecal fat. The three dogs were found to absorb uniformly 96.5 to 97.9 per cent of the food fat.

Vitamin D remained circulating in the blood for about 5 months following the administration of delsterol and of irradiated ergosterol but for only about 3 months in the case of the tuna liver oil. The elevation of serum calcium was both greater and longer sustained following the giving of the irradiated ergosterol than in the other two cases. The hypercalcemia caused by the tuna liver oil especially was mild and transitory. Warkany and Mabon (10) found wide variation in the vitamin D of the serum of human subjects, both children and adults. The method (11) was some-

TABLE I

*Effect of One Moderate Dose (20,000 Units per Kilo) of Vitamin D upon Serum Calcium and Vitamin D Content of Serum and Feces of Dogs*

Days after dosing	Serum Ca and vitamin D*						Fecal vitamin D shown by line test in rats†					
	Dog 328, delsterol		Dog 331, irradiated ergosterol		Dog 330, tuna liver oil		Dog 328, delsterol		Dog 331, irradiated ergosterol		Dog 330, tuna liver oil	
	Ca	Line test	Ca	Line test	Ca	Line test	(1)	(2)	(3)	(4)	(5)	(6)
	mg. per cent		mg. per cent		mg. per cent							
0 (Before dose)	9.5	1.5	10.8	1.0	10.4	1.0	0	1/21	0	1/12	0	1/16
4 (Hrs.)	15.4	3.0	14.3	3.0	12.1	2.5	3.0	1/11	2.0	1/8	2½	1/10
9 "	14.1	3.0	12.3	2.5	12.3	3.0						
1	10.3	4.0	14.6	2.0	14.2	3.0	0	1/6	0	1/11	0	1/11
2							0	1/4	0½	1/13	0	1/6
3							0	1/7	0	1/3	0	1/7
4-11		3.0	14.5	1.5	11.2	3.0	0	1/6	0	1/5	1-0	1/5
12-19	10.7	2.0	13.9	2.5	10.6	3.0	0	1/5	0	1/4	0	1/3
20-26		3.0	14.2	3.0	11.5	1.0	0	1/6	0½	1/5	0	1/4
27-33	11.5	2.0	12.5	2.5	9.8	0						
34-40		3.0		0	10.5	2.0						
41-47	10.6	4.0	13.4	2.5	12.4	2.5						
48-54	11.6	3.0	12.7	2.5	12.2	2.0						
55-61	8.9		12.2		11.0							
62-68	10.5	3.0	12.8	4.0		2.5						
69-76	11.2		10.2									
77-83	11.5	2.0		2.5	9.4	3.0	0	1/2	0	5/12	0	1/2
84-90	9.6		12.5		11.8							
91-97	10.1		11.0		11.4							
98-104	9.8	2.0	10.5	2.5	10.0	3.0						
105-111	11.3	2.0	11.3	0.5	8.8	0						
112-118	11.5	1.5	11.5	3.5	11.8	0.5						
119-132	10.5	2.5	11.2	3.0	11.5	0						
133-139	11.3	1.5	11.3	2.5	10.5	1.0						
140-146		2.5		2.0		0						
147-153		2.5		2.0	10.8	0						
154-160		2.5		1.0		0						
161-167		Trace		0.5		0						
168-173		0		3.0		Trace						
174-180		0		0.5		0						

\* Shown by the line test in rats fed 3 cc. of dog serum.

† The figures shown in Columns 2, 4, and 6 represent the fractions of the total fat extract of the feces which were fed to individual rachitic rats.

what like that used in this study but an attempt was made to determine the actual level of vitamin in the serum. For 89 subjects they found the



average value 116.4 units per 100 ml., with a range of 66 to 165. The quantity present in the dog serum used in this study must have been near 100 units per 100 ml. of serum during the periods when the presence of the vitamin was demonstrable. In a later study of six adult arthritic patients (12) given from 50,000 to 500,000 units of vitamin D daily over a long period the serum was found to contain 9000 to 13,000 units per 100 gm. and to maintain a very high level for several months after the treatment was stopped.

These results are of interest in connection with the vitamin D-Stoss or loading technique introduced since 1936 by German clinicians for the prevention of rickets in infants. Harnapp (13), Vollmer (14), Zelson (15), and others have reported success in curing and preventing rickets by the use of single massive doses of vitamin D<sub>2</sub> or D<sub>3</sub> either parenterally or by mouth. The economy and certainty of the procedure are advanced as reasons for preferring it to the administration of small daily doses over long periods of time. From 3 to 15 mg. of irradiated ergosterol, or vitamin D<sub>3</sub>, presumably 120,000 to 600,000 u.s.r. units, administered in one dose, have been reported as a safe and effective antirachitic treatment.

Few experimental observations have been made, however, and nearly all the reports concern only clinical freedom from rickets. Completeness of absorption, amount and site of storage, and pathological effects, immediate or remote, of these large single doses need to be established. Windorfer's (8) study of absorption indicated that less than 10 per cent of the vitamin was excreted in the feces of six rachitic infants, all within 3 days after administration of 10 to 15 mg. of vitamin D<sub>2</sub> and in one case on which autopsy was performed less than 8 per cent of the dose was found in the tissues. Vollmer (16) made a similar study of the tissues of two children which had received 200,000 units of vitamin D<sub>3</sub> per kilo of weight and was able to find very little stored vitamin.

Coppens and Metz (17) detected vitamin D in the liver, adrenal cortex, lungs, blood, spleen, mesentery, and in two out of four cases, in the kidneys of dogs 10 hours after the feeding of irradiated ergosterol, but none in heart, brain, skin, and pancreas. If the animals were allowed to survive longer, there were progressive losses in the liver until, after 2 days, there was none left in that organ. Metz and Coppens (18) postulated the existence of an enzyme in the lungs which was capable of decomposing vitamin D in the presence of oxygen, since the vitamin disappeared rapidly from lung tissues originally rich in vitamin D if kept in an incubator for 24 hours, but not if the lungs were kept in an atmosphere low in oxygen at low temperature. Only a small amount of the ingested vitamin D was stored in the blood and liver of growing calves fed cod liver oil concentrate or irradiated yeast (19), but after a single large dose of irradiated ergosterol fed to male adult

rabbits vitamin D was found (3) in the brain for 1 to 2 weeks, in the red blood corpuscles for 5 to 6 weeks, in the skin for 6 to 8 weeks, in the kidney for 6 to 9 weeks, and in liver and blood for 12 weeks.

*Tissue Storage of Vitamin D*—The next step taken therefore in this study was the administration of a large dose of the vitamin to the dogs in order to discover the place and amount of storage of excess vitamin D. When the same three dogs previously studied were 12 to 14 months old, they were given orally one dose of 200,000 units of vitamin D per kilo. One animal was given irradiated ergosterol and the two others delsterol.

Blood samples taken before and at intervals after the dose were examined for serum calcium, inorganic phosphorus, and vitamin D. Daily collections of feces were made and the dogs sacrificed 3 days after the administration of the vitamin dose. The dogs were killed by an intravenous injection of sodium amytal and exsanguinated by draining the carotid artery, and the following organs were removed for vitamin D assay: spleen, stomach, small intestine, large intestine, liver, kidney, heart, lungs, brain, muscle, skin, and subcutaneous fat. Non-saponifiable extracts were made after the method previously described, except that the non-saponifiable matter of each collection was dissolved in 1 gm. of corn oil.

The dog which received irradiated ergosterol showed no visible untoward effect of the large dose on the 1st or the 2nd day after the administration, but responded with a little vomiting on the 3rd day. The dogs which received the delsterol were more affected. They vomited on the 1st day, had diarrhea, and showed little activity. By the 3rd day they were in an advanced state of lassitude and weakness, had rapid respiration, running eyes, and refused to take water or food.

The results of vitamin D assay of the tissues are summarized in Table II and changes in serum calcium and inorganic phosphate in Table III.

Obviously there is an initial drop within the first 4 hours followed by a rise to a peak in 12 hours in both serum calcium and inorganic phosphate. This rise was greater, occurred somewhat later, and was longer sustained for calcium but less for phosphate in the case of the irradiated ergosterol than in those of the irradiated animal sterol. This is consistent with the serum calcium findings of the earlier experiment (Table I). Vitamin D in excess of 300 units per 100 gm. of serum was found in each case in blood samples taken within 45 minutes after the massive dose was given and was maintained thereafter, although serum samples withdrawn the day before were negative.

Vitamin D was present in the feces of all three dogs on the 1st and 2nd days after the dose was given and on the 3rd day in the case of the two dogs which had received delsterol but not in the case of Dog 331 which had received irradiated ergosterol. Possibly the absence of diarrhea in the

TABLE II

*Vitamin D Content of Tissues of Dogs Given Single Massive Dose (200,000 Units per Kilo) of Vitamin D<sub>2</sub> or D<sub>3</sub>*

Tissue	Total weight of fresh tissue taken		Weight of diluted unsaponifiable extract		Amounts fed		Vitamin D per gm. fresh tissue, U.S.P. units	
	Dog 331	Dog 328	Dog 331	Dog 328	Dog 331	Dog 328	Dog 331	Dog 328
	gm.	gm.	gm.	gm.	gm.	gm.		
Kidney	50	82	6.9	13.7	0.5 1.0 0.1	0.2 0.1 0.05	3-5	8
Heart	70	92	10.0	15.3	0.5 1.0 0.1	0.2 0.1 0.05	5	3-5
Small intestine	204	235	32.2	39.2	1.0 0.1	0.5 0.2 0.1 0.05	1.5	5
Large "	39	39	5.2	6.5	1.0 0.1	1.0 0.5 0.2 0.1	0.5-5.0	3-5
Liver	287	377	46.3	62.9	0.5 1.0 0.1	0.2 0.1	1.6	3-5
Lung	69	97	10.1	16.1	0.5 1.0 0.1	0.2 0.1 0.05	0.7-1.0	3-5
Brain	73	77	11.0	12.8	0.5 1.0 0.2 0.1	0.5 0.2 0.1	0.7-1.0	3-5
Spleen	35	37	4.1	6.2	1.0 0.1	0.5, 0.2 0.1	0.4-1.0	3-5
Muscle	32	39	4.0	6.5	1.0 0.2	0.5 0.2 0.1	0.5-1.0	<3
Stomach	86	80	12.7	13.3	1.0 0.2	1.0 0.5	0.7	0
Abdominal fat	34	128	4.8	21.4	1.0 0.5	1.0 0.5	0.3-0.5	<1
Skin and hair	237	322	39.2	53.7	1.0 0.2	0.5 1.0	0.5-1.5	0

latter may account for this difference. Similarly the total excretion of both calcium and phosphate was little affected during the 2 days after the massive dose of irradiated ergosterol was given to Dog 331 but in the case

of the delsterol treatment of Dog 330 an immediate increase in calcium excretion occurred, followed on the 2nd day by a similar increase in phosphate excretion (Table IV).

TABLE III  
*Serum Calcium and Phosphorus of Dogs Given One Massive Dose of Vitamin D (200,000 Units per Kilo)*

The values are expressed in mg. per 100 ml. of serum.

Hrs. after dosing	Dog 331, irradiated ergosterol		Dog 330, delsterol		Dog 328, delsterol	
	Ca	P	Ca	P	Ca	P
Before	10.9	2.5	11.4	3.2	8.0	3.0
0.50				4.2		
0.75	11.1	2.4				
1.0			11.1	3.6	11.5	3.0
1.25		3.4				
2.0				3.6		
4.0			8.4	3.6	13.8	3.1
4.5	8.5	3.5				
7.0						7.7
8.0			13.9	7.1		
9.0	16.0	6.4				
24.0	15.9	4.3	13.0	4.4		5.7
48.0	14.0	4.9	9.5	4.8	12.2	4.2
72.0	11.4	3.1	9.8	3.6	12.0	3.6

TABLE IV  
*Fecal Excretion of Ca and P after Administration of Massive Dose of Vitamin D (200,000 Units per Kilo)*

Dog No.	Body weight	Vitamin given	Collection period	Fecal vitamin D	Total excretion	
					Ca	P
	kg.				mg.	mg.
331	9.3	Irradiated ergosterol	Day before dosing	0	187	1497
330	8.2	Delsterol		0	160	1139
331	9.3	Irradiated ergosterol	1st day after dosing	++	131	1274
330	8.2	Delsterol		++	402	1148
331	9.2	Irradiated ergosterol	2nd day after dosing	++	213	1048
330	8.0	Delsterol		++	176	1480
331	8.8	Irradiated ergosterol	3rd day after dosing	0	83*	324*
330	7.9	Delsterol		++	83*	208*

\* Feces only; urine lost.

The greatest deposits of vitamin D appeared to be in the kidney, heart, and intestines, and the least in stomach, skin and hair, fat, and muscle. The liver, lung, and brain were intermediate and appeared to be capable

of only moderate storage of the vitamin. This confirms the finding of Metz and Coppens (18) of the disappearance of the vitamin from dog livers and lungs within 3 days of administration and of Vollmer (16) on its absence from liver and brain of a child similarly dosed.

The maximum vitamin content of the dog tissues mentioned in Table II was determined for both dogs in most cases. None of the tissues contained more than 5 units per gm. of fresh weight, except for the kidney of Dog 328, 8 units per gm., thus accounting for less than 60,000 units of the total dose of 2,400,000 units fed, in the case of Dog 328 which weighed 12 kilos. Some tissues other than those examined must therefore have carried the major portion of the stored vitamin, unless the blood was the repository. Maximum values for the serum were not determined but even if such values as those mentioned by Warkany *et al.* (12) prevailed (9000 to 13,000 units per 100 ml.) less than 100,000 units would be thus accounted for, and approximately 10 per cent of the whole dose in all the tissues estimated. In the case of Dog 331 the amount of vitamin stored in the tissues was usually less than was found in Dog 328, and again the total accounted for was less than 10 per cent of the vitamin administered.

The state of our knowledge as to the place of storage or of destruction of excesses of vitamin D is unsatisfactory. Only four human cases, children 1 to 4 years of age, given 200,000 units per kilo, have been studied, one by Windorfer (8) and three by Vollmer (16). The findings were inconsistent, as noted previously. Before acceptance of the vitamin D-Stoss therapy for rickets can be advocated, more must be known on this and the related problem of the sequelae of long continued hypercalcemia. In any case it would appear that irradiated ergosterol (vitamin D<sub>2</sub>) is more likely to induce more prolonged and continuous elevation of serum calcium level than does irradiated cholesterol (vitamin D<sub>3</sub>).

All of these observations tend to confirm the conclusion suggested in an earlier study (1) with rats that irradiated animal sterol (delsterol) is more rapidly absorbed and also more rapidly excreted, immobilized, or destroyed when fed in excess than is irradiated ergosterol. Again the vitamin D of tuna liver oil appeared to be milder and more transitory in its effects than either of the other forms.

#### SUMMARY

1. When single doses of 20,000 units of vitamin D per kilo of body weight were given in the form of delsterol (activated animal sterol), irradiated ergosterol, or tuna liver oil to three young dogs depleted of vitamin D for 2 months, there was in no case an appreciable amount of vitamin D excreted in the feces after the 1st day.
2. Vitamin D was present in the blood for about 100 days when tuna

liver oil was given, and for about 5 months when either delsterol or irradiated ergosterol was the source of vitamin D.

3. Delsterol caused an immediate rise in blood calcium, which fell to normal within a week. Irradiated ergosterol caused a prolonged rise in blood calcium lasting for about 2 months, while tuna liver oil caused only a mild and transitory hypercalcemia.

4. This single dose protected the dogs against rickets and allowed optimum growth for the 12 to 14 months of observation following its administration.

5. A single massive dose (200,000 U.S.P., per kilo of body weight) of vitamin D administered to these three dogs when they were 12 to 14 months old, as delsterol to two and as irradiated ergosterol to the third, produced severe and immediate prostration in the former and less obvious disturbance in the latter case.

6. Vitamin D appeared in the feces after administration of the dose for 2 days only in the case of the irradiated ergosterol but for 3 days preceding sacrifice of the animals given delsterol.

7. Calcium excretion was increased at once by the delsterol but not by the irradiated ergosterol but serum calcium was raised more markedly by the latter.

8. Vitamin D was found in the serum and most of the tissues examined but in markedly limited amount. No tissue of either dog contained more than 5 units per gm. of fresh tissue, except for the kidney in one dog given delsterol, which had 8 units per gm. Less than 10 per cent of the vitamin given in either form can be reasonably accounted for.

9. The bearing of these findings on the value and safety of antirachitic prophylaxis for infants through single massive doses of vitamin D is discussed.

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# MECHANISM OF VITAMIN D ACTION IN DOGS SHOWN BY RADIOACTIVE PHOSPHORUS\*

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Vitamin D in small amounts has been shown to aid calcium and phosphorus assimilation in the animal body, but the mechanism underlying this process is not clearly understood. Harris and Innes (1) concluded from their observation that vitamin D increases the net absorption of Ca or P or both from, or decreases the excretion into, the gut, thereby increasing the apparent solubility of Ca and P in the blood and facilitating calcification in certain sites. Taylor, Weld, and Sykes (2) suggested that vitamin D promotes Ca and P utilization through stimulation of hormone production by the parathyroid glands. There is a lack of conclusive evidence for this relationship. Nicolaysen (3) has put forward the view that the action of vitamin D in the gut is confined to a direct effect on the absorption of calcium, thus only indirectly affecting the absorption of phosphorus. Recent experiments with the radioactive isotope of phosphorus also indicate that increasing the net absorption of phosphorus is probably not the outstanding action of vitamin D. Dols *et al.* (4, 5) have reported from experiments with radioactive phosphorus that vitamin D appears to have no characteristic action on the absorption or the excretion of the labeled phosphorus in the gut of the rachitic rat. They found P metabolism of the bone of rachitic chicks as a whole to be more intense than that of normal birds. Furthermore the radioactive P was concentrated more rapidly in the epiphyses than in the diaphyses. Cohn and Greenberg (6) concluded from their similar experiment with rachitic rats that vitamin D "acts to aid the conversion of organic to inorganic phosphorus, and that the transfer of phosphorus from blood to the organic fraction of bone is independent of vitamin D." Morgareidge and Manly (7) found that the vitamin D administered to rachitic rats had no influence on the entrance of P into the blood or into the diaphyseal portion of the tibia, although it produced healing in the metaphyses, as shown by the positive line test, which occurred concomitantly with a significant increase in the content of radioactive P. These results supported their conclusion that vitamin D has, at least for one of its actions, a direct influence on the metabolic factors controlling calcification and its action is not limited to

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the control of intestinal absorption of the elements concerned in calcification.

In the present experiment, a single large dose of vitamin D was fed along with the radioactive P in order to produce striking effects of the vitamin for the purpose of observing its influence on the P metabolism.

#### EXPERIMENTAL

The radioactive phosphorus used as the indicator,  $P^{32}$ , was prepared by the bombardment of red  $P^{31}$  by accelerated deuterium ions in the Berkeley cyclotron.<sup>1</sup> It has a half life of 14.5 days and emits  $\beta$ -rays. A 1 per cent  $Na_2HPO_4$  solution was prepared from the radioactive phosphorus and fed as such.

Three adult pure bred cocker spaniel dogs, a bitch and her two daughters, were used in this experiment. They had been subsisting for more than 1 year on a stock dog diet.<sup>2</sup>

Dog 301, 3 years old, which served as the control, received 375 i.u. of vitamin D daily from cod liver oil. When 14 months old, Dog 330 received a single dose of 200,000 i.u. per kilo of body weight of irradiated ergosterol,<sup>3</sup> and Dog 331, when 12 months old, received the same massive dosage in the form of delsterol.<sup>4</sup> The oils were fed by mouth to Dogs 330 and 331 and immediately following the dosing all three animals received by stomach tube 1 cc. per kilo of body weight of the radioactive phosphate solution. Blood samples were then taken at frequent intervals for the purpose of following Ca, inorganic P, and radioactive P levels of the serum. Daily urine and feces collections were made for 3 days for the study of Ca, P, and radioactive P excretion. The dogs were anesthetized 72 hours after the administration of the dose by an intravenous injection of sodium amytal and exsanguinated by draining blood from the carotid artery, and the following organs were removed: spleen, stomach, small intestine, large intestine, liver, kidneys, adrenals, ovaries, heart, lungs, brain, a sample of leg muscle, skin, and a layer of fat underneath the skin of the dorsal side. The femur and tibia were also saved.

<sup>1</sup> We acknowledge with gratitude our debt to M. D. Kamen of the Radiation Laboratory, University of California, and to M. Murayama for the preparation of the radioactive phosphate.

<sup>2</sup> The stock diet consisted of granulated sugar 20.9 per cent, corn-starch 8.4, raw casein 45.8, fat 10.0, Salts 5 (8) 2.4,  $CaCO_3$  1.5, wheat germ 5.0, and brewers' yeast 6.0 (see Morgan, A. F., and Shimotori, N., *J. Biol. Chem.*, in press).

<sup>3</sup> The irradiated ergosterol, 1,000,000 i.u. per gm., was furnished us by C. E. Bills of Mead Johnson and Company, Evansville, Indiana.

<sup>4</sup> The delsterol, irradiated animal sterols, 300,000 i.u. per gm., was furnished us by J. Waddell of E. I. du Pont de Nemours and Company, Inc., New Brunswick, New Jersey.

After the total fresh weights of the organs were obtained, the organs were ground through a food chopper and approximately equal aliquots of the tissues were weighed into 10 ml. Coors ashing capsules, dried, and ashed in an electric muffle furnace at 500°. The ashes were moistened with a few drops of distilled water in order to distribute them as evenly as possible over the bottom of the capsules and dried on a hot-plate. The femurs were extracted twice with an alcohol-ether mixture for about 10 hours, then dried, and ashed as above. The ash was dissolved in dilute HCl and the volume brought to 100 ml.; 2 ml. aliquots were measured into capsules and evaporated to dryness. To determine the amount of radio-



FIG. 1. Radioautographs of split tibias of dogs given radioactive P without (control; center tibia in photograph) and with a massive dose of irradiated ergosterol (left-hand in figure) or cholesterol (right-hand in figure).

active P in the inorganic form in the blood serum, a sample of the solution left after the inorganic P determination of Fiske and Subbarow (9) was measured into a capsule and evaporated to dryness.

The radioactivity measurements were made with the Lauritsen electroscope. The rate of decay of a standard consisting of 0.001 ml. of the radioactive phosphate solution was followed. This served to establish the radioactivity of the standard at any given time. The activity of the samples was then determined relative to that of the standard, all being expressed in percentage of the dose administered. The radioactivity was measured in terms of divisions per second of discharge in the electroscope, from which the background was subtracted. The value thus obtained

from a sample was divided by the corresponding value obtained for the standard at the same time. This resultant value multiplied by the ratio of ml. administered to ml. in the standard indicated the per cent of the fed phosphate present in the sample.

After radioactivity measurements were made, the ashes were dissolved in dilute HCl, the solution brought to volume, and total phosphorus determined after the method of Fiske and Subbarow (9).

In order to observe the distribution of radioactive P in the bone, radioautographs were taken of the tibia (Fig. 1). The tibia was sawed in half and the cut surface was held pressed upon a sheet of Agfa "no screen" x-ray film. After 5 days exposure, the film was developed. The greatest effect of the exposure took place where the radioactive P was present in the largest concentration.

#### DISCUSSION

The dogs which received the large doses of vitamin D showed no gross untoward effect except some diarrhea and vomiting on the 3rd day following the feeding. They both lost some weight, about 1 pound each, in the 3 day period.

The labeled inorganic phosphorus of the blood disappeared rapidly, most of it being diffused from the blood in 2 hours. There was no marked difference in the rate of this disappearance between the control dog and the dogs which received the large doses of vitamin D, although there was a definite hyperphosphatemia in the latter several hours after the administration of the vitamin (Table I). It is clear that, since the vitamin had no measurable effect upon the radioactive P fraction of the serum P, increase in the rate of absorption of P from the gut cannot be looked on as a primary part of the vitamin action. The rise in total inorganic P of the serum which is usually reported following the ingestion of vitamin D and which was seen in the dogs given the vitamin in this experiment must be due to mobilization of P from the tissues rather than from the intestine. Morgareidge and Manly (7) using similar technique on rats also found that vitamin D had no influence upon the amount of labeled P in the serum.

The hypercalcemia, which was more marked and longer sustained in the dog given irradiated ergosterol than in that given delsterol, might also by analogy be ascribed to tissue rather than food sources.

The irradiated ergosterol caused a 2-fold or more increase in the urinary total phosphorus excretion and the delsterol a smaller increase, but the radioactive phosphorus excretion was lower in the experimental than in the control (20.0 and 17.8 as compared with 23.3 per cent) (Table II). This indicates that both vitamins D<sub>2</sub> and D<sub>3</sub> promoted excretion of urinary

TABLE I  
*Effect of Vitamin D on Serum of Dogs Given Radioactive P*

Time after administration of vitamin	Dog 301. No vitamin D			Dog 330. Given irradiated ergosterol			Dog 331. Given delsterol		
	Ca	P	Radio-active P	Ca	P	Radio-active P	Ca	P	Radio-active P
hrs.	mg. per cent	mg. per cent	per cent total dose per 100 ml.	mg. per cent	mg. per cent	per cent total dose per 100 ml.	mg. per cent	mg. per cent	per cent total dose per 100 ml.
Before	9.5	4.4		10.9	2.5		11.4	3.2	
0.33		4.4	1.168					4.2	1.128
0.50									
0.75				11.1	2.4				
1.00		4.4	1.008				11.1	3.6	0.691
1.25					3.4	0.207			
2.00								3.6	0.412
2.50		4.4	0.308						
4.00		4.2	0.183				8.4	3.6	0.161
4.50				8.5	3.5	0.095			
8.00			0.135				13.9	7.1	0.150
9.00				16.0	6.4	0.109			
24.00			0.077	15.9	4.3	0.053	13.0	4.4	0.070
48.00			0.079	14.0	4.9	0.038	9.5	4.8	0.040
72.00		4.0	0.043	11.4	3.1	0.028	9.8	3.6	0.029

TABLE II  
*Urinary Excretion of Total and Radioactive P As Affected by Vitamin D*

Dog No.	Days after administration of vitamin and $P^{32}$	Ca	P	Radioactive P	Specific activity
		mg.	mg.	per cent of dose	$\times 10^{-4}$
301	1	11	441	15.5	3.51
	2	81	412	4.4	1.07
	3	20	432	3.4	0.79
Total .....		112	1285	23.3	5.37
331	0	21	447		
	1	244	445	8.8	1.97
	2	53	1003	7.3	0.73
	3			3.9	
Total.....				20.0	
330	0	35	586		
	1	27	419	10.6	2.53
	2	135	733	4.7	0.64
	3			2.5	
Total.....				17.8	

phosphorus, but that this increased excretion must have originated from sources other than recently ingested phosphorus. The urinary Ca was also increased on the 1st day by the delsterol and on the 2nd by the irradiated ergosterol. There was a marked decrease in fecal P and Ca corresponding roughly with the urinary increases.

The results obtained on the feces are strikingly different for the two forms of the vitamin (Table III). In both cases vitamin D caused a progressive decrease in the total fecal P, but the excretion of the radioactive P was definitely lower in the dog which received delsterol and higher in

TABLE III  
*Fecal Excretion of Total and Radioactive P As Affected by Vitamin D*

Dog No.	Days after administration of vitamin and P <sup>32</sup>	Ca	P	Radioactive P	Specific activity
		mg.	mg.	per cent of dose	$\times 10^{-4}$
301	1	216	930	9.6	1.03
	2	229	778	1.2	0.15
	3	225	810	0.5	0.07
Total .....		670	2518	11.3	1.25
331	0	139	692		
	1	158	703	3.4	0.49
	2	123	477	0.9	0.18
	3	83	208	0.2	0.10
Total .....		364	1388	4.5	0.77
330	0	152	911		
	1	104	855	29.5	3.33
	2	78	315	6.5	2.07
	3	83	324	0.5	0.15
Total .....		265	1494	36.5	5.55

the dog which received the irradiated ergosterol than in the control animal. The total radioactive P excreted during the 3 day period was 24.5 per cent by the dog given delsterol, 34.6 per cent by the control, and 54.4 per cent by the dog given irradiated ergosterol.

The total P and Ca excretion was not significantly altered in the 3 days, since with the marked increases in urinary P and Ca there were decreases in the corresponding fecal excretion. There appeared therefore to be no significant effect of the vitamin on the net retention of total P. This accords with the view of Nicolaysen (3).

The ratio of total fecal P to urinary P remained nearly the same, 2.1 to 1.9, in the control animal and the corresponding ratio of per cent of  $P^{32}$  to total P in feces and urine (specific activity) declined throughout the 3 days, 0.29, 0.14, 0.09. The corresponding ratios for total excreted P of the dog given delsterol were 1.6 and 0.47 and for  $P^{32}$  were 0.25 and 0.25 on the 1st and 2nd days, thus indicating that the apparent increase in P absorption may be due to failure of reexcretion into the gut or to increased tissue P mobilization, but not to decreased reabsorption by the renal tubules. The dog given irradiated ergosterol, on the other hand, excreted fecal and urinary total P in the ratios 2.0 and 4.3 and  $P^{32}$  in the ratios 1.3 and 3.2 on the 2 days following the administration of the vitamin. The ratio  $P_f:P_u$  on the 2nd day was more than doubled, but the ratio  $P_f^{32}:P_u^{32}$  was increased to 4 times the value in the control on the 1st day and 23 times that on the 2nd day. Again there appears no reason to assume failure of reabsorption by the kidneys but instead either failure of intestinal absorption or increased reexcretion into the intestine.

It is interesting to note the total P excreted during the 1st and 2nd days by the three dogs, 2.56, 2.63, and 2.22 gm. respectively for Dogs 301, 331, and 330, and the fecal P 1.71, 1.18, and 1.17 gm.; yet the proportion of radioactive P in the feces was 11.2, 4.5, and 36.5 per cent of the dose administered. The effects of both forms of vitamin D appear therefore to be identical so far as entry of newly ingested P into the serum is concerned and also its excretion in the urine, but to differ sharply in regard to excretion from the intestine. Nevertheless the apparent effects of the two forms of vitamin D are identical if total P excretion is examined.

The large dose of vitamin D caused lowered retention of the radioactive P of the soft tissues expressed in terms of activity per gm. of dry weight (Table IV). Delsterol caused a decrease in the specific retention in almost all the organs studied, but the decrease was less marked or not present in the case of kidney, large intestine, brain, small intestine, and lungs, but was particularly great in the subcutaneous fat and the skin, stomach, spleen, and liver. Irradiated ergosterol caused a marked lowering of radioactive P in all the tissues investigated and, as in the case of delsterol, the decrease was most marked in the fat and the skin, stomach, spleen, and liver.

On the other hand, in the femur, the radioactive P turnover was increased up to almost 2-fold in the dogs which received the vitamin, the increase being greater in the case of irradiated ergosterol. The bone radioautographs showed that the vitamin D intensified the P metabolism in the spongy portion of the bone, since the greatest deposition of  $P^{32}$  was in the metaphyseal and the epiphyseal regions of the bone.

The specific activity of the organs, expressed as the per cent radioactive

P ingested per mg. of total P in the dry tissue again indicated that the large dose of vitamin D in both forms caused in general a lowered specific activity of the soft tissues. On the other hand, it increased the specific activity of the femur, more P atoms in the latter being replaced by the active P.

Irradiated ergosterol exerted a much more marked and consistent effect both on the specific retention of the soft tissues and of the femur than did the delsterol. The decrease in the specific retention was most pronounced in the case of fat and skin, in which the values were lowered to as little as

TABLE IV

*Distribution of Radiophosphorus in Tissues of Dogs without Vitamin D (Dog 301) and with a Large Dose of Vitamin D<sub>2</sub> (Dog 330) and Vitamin D<sub>3</sub> (Dog 331)*

Tissue	Radioactive P, per gm. dry tissue			Total P in dry tissue			Radioactive P, specific activity		
	Dog 301	Dog 330	Dog 331	Dog 301	Dog 330	Dog 331	Dog 301	Dog 330	Dog 331
	per cent of dose	per cent of dose	per cent of dose	per cent	per cent	per cent	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$
Spleen .....	0.067	0.036	0.042	1.08	0.86	0.75	6.2	4.2	5.5
Stomach.....	0.050	0.024	0.035	0.95	0.62	0.84	5.2	3.8	4.1
Small intestine.....	0.089	0.065	0.084	1.23	1.17	1.41	7.2	5.5	6.0
Large " .....	0.054	0.037	0.059	0.79	0.74	0.92	7.0	5.0	6.4
Liver.....	0.088	0.048	0.066	0.97	0.87	0.99	9.0	5.5	6.7
Kidney.....	0.071	0.052	0.074	1.09	1.15	1.37	6.5	4.5	5.4
Adrenals.....	0.069	0.041	0.064	1.11	0.88	1.09	6.3	4.6	5.8
Ovaries.....	0.054	0.031	0.042	1.06	0.77	1.14	5.1	4.0	3.7
Heart.....	0.051	0.031	0.042	0.86	0.90	0.99	6.0	3.4	4.2
Lungs.....	0.059	0.049	0.056	1.02	1.23	1.13	5.8	4.0	4.9
Brain.....	0.008	0.006	0.009	1.43	1.46	1.60	0.5	0.4	0.5
Muscle.....	0.034	0.024	0.023	0.62	0.75	0.77	5.5	3.2	3.0
Fat.....	0.004	0.001	0.001	0.07	0.03	0.02	6.0	3.3	5.0
Skin and hair .....	0.018	0.003	0.007	0.38	0.62	0.20	4.7	0.5	3.5
Femur ash.....	0.036	0.074	0.064	16.9	16.7	16.3	0.2	0.4	0.4

one-fourth and one-sixth, respectively, of the control values. The specific retention by the bone was increased 86 per cent and 109 per cent by delsterol and irradiated ergosterol, respectively, the greatest activity being concentrated in the epiphyseal and the metaphyseal regions, as is evident from the tibia radioautograph. It appears that vitamin D causes an increased uptake of the labeled P in the bones at the expense of the soft tissue P, particularly that of the skin and fat.

This finding is consistent with the view advanced by Cohn and Greenberg (6) that under the influence of vitamin D a flow of phosphate takes place from the soft tissues to the bones. Likewise their finding with rats

as to the order of affinities of the labeled P for the tissues is substantially confirmed by this experiment on dogs. The order of decreasing proportions of the total dose of labeled P found in the tissues is bone, liver, small intestine, kidney, adrenals, spleen, lungs, large intestine, ovaries, stomach, muscle, skin, brain, fat. On the other hand the order of specific P turnover is liver, small and large intestines, kidney, spleen, adrenals, heart, stomach, lungs, brain, muscle, fat, skin, ovaries, bone. This P turnover was increased in bone but decreased in all other tissues except skin by vitamin D<sub>2</sub> and except brain by vitamin D<sub>3</sub>. The latter may be a significant finding in view of clinical reports of hypervitaminotic symptoms in infants as well as the differences reported for brain storage of vitamins D<sub>2</sub> and D<sub>3</sub> (10, 11).

The question raised by the diverse conclusions of Schneider and Steenbock (12) and Day and McCollum (13) as to the mechanism of vitamin D action is only partly answered by these findings. The former authors concluded that, under the influence of the vitamin on low P intake, P was stripped from soft tissues and added to bone, and the latter that the reverse condition obtained. Earlier experiments with rats in this laboratory (14) as well as in others pointed to the grave importance of the level of Ca and P in the diet for bone growth, vitamin or no vitamin, except in the low P cereal diets. The radioactive P and total P circulation, deposition, and excretion here reported appear to indicate no necessary relation between the levels of total P and radioactive P in the serum under the influence of the vitamin, nor in the urinary excretion, except for depression instead of increase in the urinary radioactive P, and diverse effects upon the radioactive P content of the feces, with no consistent relation to the total P of the feces.

Again the content of radioactive P in soft tissues was decreased and that in bones increased by the vitamin but these changes bore no relation to the total P of these tissues. The only conclusion possible is that newly ingested food P is not the source of the hyperphosphatemia, increased urinary, nor decreased fecal P output due to vitamin D activity. Nor can it be said that food P is diverted from soft tissues to bone, but only that P turnover in the former is depressed and stimulated in the latter.

If the increased serum and urinary total P, in contrast with radioactive P, arose from the stripped diaphyses, the heightened P turnover of epiphyses would still be possible. In any case no support for the improved intestinal absorption theory of vitamin D action is furnished by this experiment.

#### SUMMARY

1. Vitamin D fed in the form of irradiated ergosterol and delsterol at the level of 200,000 i.u. per kilo of body weight had no significant effect



on the rate of disappearance of the labeled inorganic phosphate from the blood stream.

2. There was a marked increase in the urinary phosphate excretion in the experimental animals, although the radioactive phosphate excretion was even less than in the control; therefore the increased phosphate must have originated from sources other than that of recently ingested phosphate.

3. The irradiated ergosterol greatly increased the fecal loss of labeled P but the delsterol acted to decrease this loss. This was the most striking difference between the effects of the two forms of vitamin D.

4. The large dose of vitamin D caused decreased P turnover in almost all the soft tissues studied, but increased to almost 2-fold the P metabolism of the femur. The P uptake was most intensified in the epiphyseal and the metaphyseal portions of the bone, the increased specific activity of the bone being due to the intensified P metabolism in these regions.

5. Vitamin D probably does not exert its therapeutic effects through improvement in intestinal absorption of P but rather by intensification of P turnover in bone with resultant hyperphosphatemia and decreased visceral P turnover.

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# BIOLOGICAL CATALYSIS OF THE EXCHANGE REACTION BETWEEN WATER AND HYDROGEN\*

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It has been known for some time that there are species of bacteria which produce and fix molecular hydrogen. The chemical reactions which are catalyzed by these bacteria may be classified as follows:

- |     |  |
|-----|--|
| (1) | $2\text{H}_2 + \text{O}_2 = 2\text{H}_2\text{O}$ |
| (2) | $\text{H}_2 + \text{CO}_2 = \text{HCOOH}$        |
| (3) | $\text{H}_2 + \text{X} = \text{H}_2\text{X}$     |
| (4) | $\text{H}_2 = 2\text{H}^+ + 2e$                  |

The enzyme which catalyzes the synthesis and decomposition of formate has been named hydrogenlyase (1). The term hydrogenase has been applied to the enzymes which catalyze the other reactions listed (2).

Farkas, Farkas, and Yudkin (3) have shown that the hydrogen evolved from formate decomposed by *Bacillus coli* in heavy water is in equilibrium with the water, for not only the concentration of deuterium in the gas phase, but the distributions of the three forms of hydrogen,  $\text{H}_2$ , HD, and  $\text{D}_2$ , are those predictable from complete chemical equilibrium between water and gas. These results indicate that *B. coli* contains an enzyme system capable of catalyzing the exchange reaction between water and molecular hydrogen. This was confirmed in separate experiments carried out without the addition of formate. It is of some interest that Green and Stickland have shown that an inert metal in a suspension of *B. coli* behaves as a reversible hydrogen electrode (4).

This paper deals with the experimental findings obtained on studying the exchange reaction between gaseous hydrogen and water, catalyzed by bacteria. This is a direct method by which the biological activation of the hydrogen molecule can be observed in the absence of organic substrate re-

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active with the hydrogen molecule. The system is therefore uncomplicated by the necessity for the activation of organic substrate.

### EXPERIMENTAL

Because some strains of *Bacillus coli* were occasionally found to be inactive as exchange catalysts, a search was made for some other species of bacterium possessing more reproducible properties. In the course of this study it was found that activated sewage sludge contains an exchange catalyst. From the sludge, pure cultures of bacteria were isolated and tested for exchange activity. One of the most catalytically active organisms was identified as *Proteus vulgaris*. Its activity has been found to

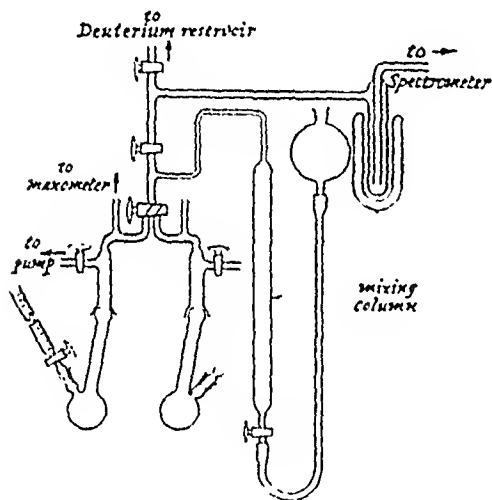


FIG. 1

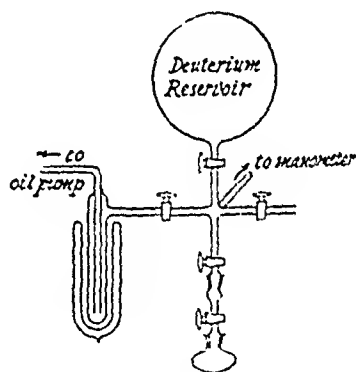


FIG. 2

FIG. 1. Apparatus for studying the exchange reaction.

FIG. 2. Apparatus for filling the exchange vessels with deuterium gas.

be constant, and, for many of the experiments to be described, *Proteus vulgaris* obtained from the Department of Bacteriology has been used.<sup>1</sup>

**Preparation of Bacterial Cultures**—Bacteria were cultured in broth of the following composition: 2 per cent Bacto-tryptone,<sup>2</sup> 0.5 per cent glucose, 0.5

<sup>1</sup> Other bacteria which were tested for exchange were *Bacillus pyocyaneus* (with and without added pyocyanine), a strain of *Bacillus welchii*, *Bacillus acidi lactici*. Only *Bacillus acidi lactici* was found active. Hemoglobin and catalase were found inactive as exchange catalysts. We are indebted to Dr. Kurt Stern for samples of pyocyanine and catalase and to Dr. Theodore Rosebury for some of the cultures of bacteria.

<sup>2</sup> A standard peptone manufactured by Difeo Laboratories, Inc.

per cent NaCl, and 0.5 per cent concentrated yeast extract.<sup>3</sup> For massive growth, broth cultures were inoculated into Roux bottles containing broth and 2 per cent agar. 18 to 24 hour cultures were washed out of the Roux bottles with distilled water, washed three times with centrifugation, and suspended in  $M/15$  phosphate buffer.

*Exchange Apparatus*—The exchange reaction was observed with two types of apparatus, shown in Figs. 1 and 2.

In the 100 cc. flasks, equipped with spherical joints, shown in Fig. 1, the contents of a single Roux bottle were suspended in 25 cc. of buffer and shaken with deuterium gas admitted from the storage bulb. Evacuation of the apparatus before the admission of deuterium was accomplished through the side arms shown. The two flasks were shaken at room temperature on the same shaft at a frequency of 150 full strokes per minute. One flask was generally used as a control.

In order to insure mixing of the gas in the shaking vessel, the mixing column shown in the diagram was used. The mercury reservoir was lowered and raised a few times, in order to expand and mix the gas in the system. The mixed gas contained between the stop-cocks of the lock was then admitted to the mass spectrometer for analysis. The total pressure of the system was decreased by 0.5 per cent by the withdrawal of a single aliquot. During the exchange run, no significant changes in pressure were noted unless certain reducible or fermentable compounds were added.

The apparatus shown in Fig. 2 was used for experiments in which the concentration of bacteria was relatively dilute, and for experiments which required constant temperature control. The oblate spherical vessels employed have a capacity of 25 cc. The half time for the exchange reaction will be shown later to depend on the volume of the system according to Equation 5.

$$(5) \quad t_{\frac{1}{2}} = 0.69V/k$$

where  $V$  is the volume of the system, and the other terms have their conventional meanings. Reactions will therefore proceed faster in the smaller flasks. At all times the total volume of suspension in these flasks was 5 cc. Evacuation was accomplished through the side arms shown, and deuterium admitted from the storage bulb. The vessels were shaken through a 1 inch stroke at a frequency of 240 strokes per minute. For analysis the flasks were fitted to a ground glass joint on the spectrometer, and the total gas content was expanded into a ballast reservoir. Samples from the reservoir were then analyzed. The temperature of the water bath was held constant to  $0.01^\circ$ .

<sup>3</sup> We are indebted to Dr. Charles N. Frey of The Fleischmann Laboratories for a sample of yeast concentrate.

*Preparation of Deuterium Gas*—Deuterium gas was prepared by electrolysis of 99.6 per cent heavy water, and was diluted with tank hydrogen to the desired atomic concentration of deuterium. The mixtures were not in equilibrium with regard to the reaction  $\text{H}_2 + \text{D}_2 \rightleftharpoons 2\text{HD}$ .

*Analyses*—Analyses of the gas phase for  $\text{H}_2$ ,  $\text{HD}$ , and  $\text{D}_2$  were carried out in a Blenkney type mass spectrometer (5, 6).<sup>4</sup> The deuterium content of water samples was measured by the falling drop technique (7).

*Effect of Protein Denaturants on Exchange Reaction*—When a washed suspension of *Proteus vulgaris*, suspended in phosphate buffer of pH 7, is shaken with heavy hydrogen, the deuterium content of the gas decreases with time, as shown in Fig. 3, Curve A. The bacteria lost all catalytic activity when autoclaved, or when dried. Attempts to prepare a dry catalyst by treatment of bacteria with cold acetone, or by freezing and drying, failed

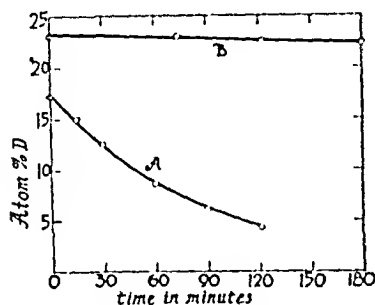


FIG. 3

FIG. 3. The rate of the exchange reaction before and after oxygen inactivation. Curve A, suspension of *Proteus vulgaris* shaken with heavy hydrogen; Curve B, with oxygen for 24 hours.

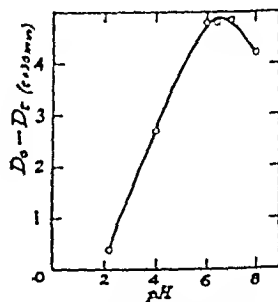


FIG. 4

FIG. 4. The effect of pH on the rate of the exchange reaction.

to yield an active preparation. A layer of toluene over the water surface inhibited the rate of exchange by 50 per cent. As the catalyst responsible for the promotion of exchange reaction shows properties typical of enzymes in general, it is presumably an enzyme or system of enzymes.

*Effect of pH on Exchange Reaction*—The effect of pH on the rate of the exchange reaction is shown in Fig. 4. The yield of a 24 hour growth of *Proteus vulgaris* from four Roux bottles was centrifuged and washed as usual. The bacteria were resuspended in 30 cc. of distilled water, from which 5 cc. aliquots were withdrawn. The aliquots were pipetted into 20 cc. of citrate-phosphate buffer, and the rate of the exchange reaction was determined after 30 minute periods. The suspensions were centrifuged at

<sup>4</sup> We assume that the ion intensities are proportional to the concentration of  $\text{H}_2$ ,  $\text{HD}$ , and  $\text{D}_2$  in the sample analyzed.

the end of the reaction, and the pH of the supernatant was determined by glass electrode measurements.

*Effect of Some Enzyme Inhibitors on Exchange Reaction*—When added to the reacting system through the side arm burette shown in Fig. 1, the following compounds were without effect on the rate of the exchange reaction: 1 per cent fluoride, 2 per cent urethane, 0.001 M iodoacetate, 0.01 M  $K_2S_2O_8$ , and 0.08 M malonate. Aerobic incubation with  $10^{-3}$  M  $Ag^+$  inhibited the exchange reaction by 85 per cent, and similar incubation with  $10^{-2}$  M  $Ag^+$  inhibited the exchange reaction completely.  $10^{-4}$  M  $Ag^+$  was without effect.

*Reversible Inactivation of Exchange Reaction*—When a suspension of *Proteus vulgaris* was shaken with oxygen for 24 hours, a partial and reversible inactivation of the exchange reaction occurred,<sup>5</sup> see Fig. 3, Curve B. The time necessary to bring about this inactivation was found to depend on the number of transfers of the bacteria through broth, oxygenation time increasing with the number of such transfers. Results were easily reproduced, however, when the bacteria were transferred from the stock culture, on an agar slant, and passed only once or twice through broth. The stock culture was inoculated into a fresh medium once a month.

Exchange activity was completely restored to the inactivated suspensions on the addition of any one of the following organic compounds: glucose, pyruvate, formate, fumarate, or succinate. In Fig. 5, Curve B, is shown the reactivation brought about by 0.04 M formate. At the point indicated by the arrow formate was added. Since the decomposition of formate by hydrogenlyase is almost completely inhibited by an atmosphere of hydrogen, the dilution of deuterium by hydrogen evolved from the formate should be negligible (1).

In Fig. 5, Curve C, is shown the reactivation of the exchange by 0.04 M pyruvate. With this, unlike the other compounds used to restore activity, there was an induction period of about 20 minutes before exchange occurred.

The effect of glucose closely resembles that due to fumarate and formate (see Fig. 5, Curve A).

The effect of 0.04 M fumarate is shown in Fig. 6, Curve A. The initial part of Curve A shows the slow decline of the deuterium concentration of the gas after the bacteria had been oxygenated for 24 hours. On addition of fumarate, an immediate increase in the rate of the exchange reaction occurred. The suspension was again inactivated by oxygenation (Curve B, Fig. 6) and was once more reactivated with fumarate. Some of the inactivation is irreversible, for complete activity was not regenerated.

The effect of succinate on the activation of the exchange is seen in Curve C of Fig. 6. In order to determine whether or not the succinic dehydro-

<sup>5</sup> We have observed a similar effect on a strain of *Bacillus coli*. Fumarate reactivated the exchange.

genase system is involved in succinate activation, the system was made 0.08 M with malonate before addition of succinate (0.04 M). The malonate neither reactivated the enzyme nor inhibited the succinate effect. Since malonate is a competitive inhibitor (8), it is possible that, even in the presence of malonate, fumarate was formed by dehydrogenation of succinate in a concentration sufficient for reactivation.

When oxygen-inactivated suspensions, to which no reactivating substance had been added, were shaken with deuterium for comparatively long periods of time, restoration of complete exchange activity slowly occurred.

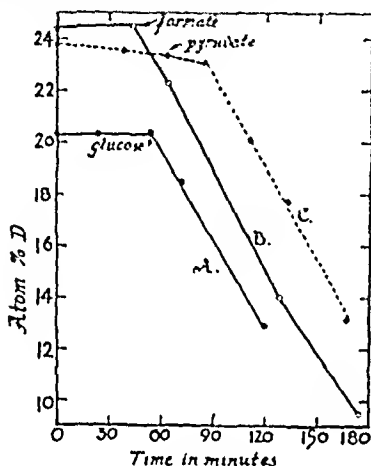


FIG. 5

FIG. 5. The reactivation of the exchange reaction by glucose, formate, and pyruvate.

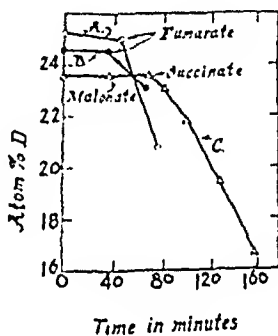


FIG. 6

FIG. 6. The effect of repeated inactivation by oxygen and reactivation by fumarate and succinate. Curve A represents the effect of 0.04 M fumarate after the bacteria had been oxygenated 24 hours; Curve B, the suspension again inactivated by oxygenation; Curve C, the system made 0.08 M with malonate before the addition of 0.04 M succinate.

*Mechanism of Exchange Activation*—At least two mechanisms must be considered to account for the inactivation of suspensions of *Proteus vulgaris* by treatment with oxygen. Either (1) an intermediate substance, necessary for the exchange reaction, is quantitatively removed by oxidation, or (2) the enzyme responsible for the exchange reaction is oxidized. Restoration of the exchange activity by the addition of formate or succinate is therefore brought about either by (1) replacement of the removed intermediate, or (2) by reduction of the oxidized enzyme. Both mechanisms can conceivably be effective simultaneously.

In the first part of this paper we have mentioned certain reactions, cata-

lyzed by bacteria, which involve the production or fixation of molecular hydrogen. All of these reactions, if reversible, with the possible exception of Equation 3, should bring about an exchange reaction between water and hydrogen. Equation 2, for example, would involve formate or  $\text{CO}_2$  as intermediate. Formate has been found to restore exchange activity to oxygen-inactivated suspensions, but the addition of  $\text{CO}_2$  to the gas phase does not restore exchange activity in the same time interval. Since the exchange reaction is activated by substances other than formate, for example succinate, the place of succinate as a possible intermediate was studied as follows: Deuteriosuccinic acid containing 47 atom per cent of carbon-bound deuterium was shaken with a suspension of *Proteus vulgaris* in ordinary water against ordinary hydrogen. No deuterium appeared in the gas phase in 48 hours. When normal succinic acid, added to a suspension of *Proteus vulgaris* in 23 per cent heavy water, was shaken with ordinary hydrogen for 48 hours, the recovered succinic acid contained 2.1 atom per cent carbon-bound deuterium. In this case the deuteriosuccinic acid might have arisen either by synthesis from substrate still retained by the suspension, or by exchange. As the concentration of deuterium in the succinic acid was less than 10 per cent of that of the water, it may be concluded that succinic acid is not a significant exchange intermediate, and that the reactivation of the exchange reaction by succinic acid was not due to the activity of the succinic acid as an intermediate. In the following discussion we will present evidence which indicates that the inactivation caused by oxygenation can be attributed to oxidation of the enzyme and removal of substances which can reduce the enzyme. The restoration of exchange activity on the addition of fumarate, succinate, etc., is accomplished by the reduction of the inactive, oxidized enzyme to the active, reduced enzyme.

That the enzyme can exist in an oxidized and a reduced state is indicated by the effect of cyanide on the exchange reaction. When a suspension of *Proteus vulgaris* was made  $10^{-3}$  M with cyanide under aerobic conditions, the suspension became completely inactive as an exchange catalyst. On the other hand, addition of cyanide to the exchanging system (which, of course, is operating under anaerobic conditions) in  $10^{-2}$  M concentration was without effect on the exchange rate. One may therefore conclude that in the presence of oxygen the enzyme responsible for the exchange reaction is in the oxidized state, and that under hydrogen, in the presence of suitable reducing agents, the enzyme is in the reduced state. Failure of iron porphyrin compounds to combine with cyanide when the iron is present in the ferrous state is well known, as, for example in peroxidase and catalase.

Further evidence that the exchange enzyme probably possesses an iron porphyrin prosthetic group has been obtained by studying the influence of carbon monoxide on the rate of the exchange reaction. In the dark, addi-



tion of 2.5 volumes of carbon monoxide for every 1 of heavy hydrogen caused complete inhibition; with equal volumes, 80 per cent inhibition occurred in the dark and 55 per cent in the presence of light.

The reversal of CO inhibition of iron porphyrin-protein enzymes by light has been reported in a number of cases, the classic example, of course, being that of the respiratory enzyme of Warburg (9). It may also be pointed out here that the exchange catalysis is not accompanied by the oscillation of the enzyme between the reduced and oxidized state, since, if this were the case, the inhibitory effect of cyanide would be detected under anaerobic, as well as aerobic, conditions.

Strong support for the "reduction" hypothesis is supplied by the effect on oxygen-inactivated suspensions of *Proteus vulgaris* of 0.01 M hydrosulfite, which completely restored exchange activity to these suspensions.

It is now possible to understand the apparently spontaneous restoration of exchange activity of oxygen-inactivated suspensions by prolonged shaking with deuterium. Even in the absence of reducing substances, there is, at the beginning, necessarily an equilibrium concentration of the reduced enzyme. It is possible that the formation of additional amounts of reduced enzyme is an autocatalytic process, which eventually produces a maximum concentration of active enzyme.

*Attempt to Separate Activity of Hydrogenlyase and Hydrogenase Systems*—The hydrogenase activity of bacteria has been demonstrated by a number of methods (10, 11). Stephenson and Stickland used as the criterion the rate of reduction of methylene blue in the presence and in the absence of hydrogen. The hydrogenlyase system is identified by measuring hydrogen output in the presence of formate. In the course of our studies it was found that *Proteus vulgaris* catalyzes both reactions. It therefore seemed necessary to determine whether or not the exchange reaction could be attributed to either of these enzymes or to both.

In her studies on the presence of the hydrogenase and hydrogenlyase systems in bacteria, Stephenson has stated that *Bacillus lactis aerogenes* contains an active hydrogenlyase, but lacks hydrogenase (2). We have found that suspensions of *B. lactis aerogenes* catalyze the exchange reaction at a rate comparable to *Proteus vulgaris*. In agreement with Stephenson's observations, the hydrogenlyase system was found to be present, as shown by the evolution from formate of hydrogen (identified in the mass spectrometer). We also found that dilute suspensions of *B. lactis aerogenes* fail to reduce methylene blue in the presence of hydrogen more rapidly than under nitrogen.

It therefore became necessary to resolve the paradox that though the hydrogen is in an activated state (since exchange and reduction of  $\text{HCO}_3^-$  by  $\text{H}_2$  occurs) it does not reduce methylene blue. The free energy change

for the reduction of methylene blue by hydrogen at pH 7 is approximately -20 kilocalories, while that for the reduction of bicarbonate ion by hydrogen to formate ion is approximately +750 calories (12). Thermodynamically the reduction of methylene blue is more readily possible than reduction of bicarbonate. Since the oxidation of leucomethylene blue is an uncatalyzed reaction, the failure to reduce methylene blue cannot be due to the absence of a methylene blue activator.

Early in our work it was found that thick suspensions of *Proteus vulgaris* reduced methylene blue much more rapidly under hydrogen than under nitrogen. If, however, the suspension of bacteria was made sufficiently dilute, the reduction of methylene blue under hydrogen failed to take place any faster than under nitrogen.

When the effect of fumarate on the reduction of methylene blue under hydrogen was studied, the following results were obtained. 25 cc. flasks (shown in Fig. 2) were prepared as follows: 2 cc. of dilute bacterial sus-

TABLE I  
Effect of Fumarate on Methylene Blue Reduction

	Reduction time			
	H <sub>2</sub> + fumarate	H <sub>2</sub> alone	N <sub>2</sub> + fumarate	N <sub>2</sub> alone
	min.	min.	min.	min.
<i>Proteus vulgaris</i> .....	4	10	30	30
<i>Bacillus lactis aerogenes</i> .....	6	24	30	30

pension of *Proteus vulgaris*, 1 cc. of 1:5000 methylene blue, and 2 cc. of M/15 phosphate buffer (pH 6.83) were shaken at 37° with hydrogen or nitrogen in the presence and in the absence of 0.04 M sodium fumarate. The reduction of methylene blue in the presence of hydrogen plus fumarate took place in 15 minutes. None of the other systems showed signs of reduction after half an hour.

However, a 5-fold more concentrated suspension gave the results shown in Table I.

It seems possible that the failure of a dilute bacterial suspension to reduce methylene blue under hydrogen is directly due to the lack of sufficient intermediate hydrogen acceptor essential to the transfer of hydrogen to methylene blue.

Stephenson concluded that *Bacillus lactis aerogenes* lacks hydrogenase, since a suspension of this bacterium failed to take up hydrogen in the presence of nitrate as well as in the presence of methylene blue. It seems reasonable to expect that the reduction of both nitrate and methylene blue requires the presence of an intermediate hydrogen acceptor.

In the group of bacteria investigated by Stephenson for hydrogenase and hydrogenlyase activity, only *Bacillus lactis aerogenes* was stated to contain hydrogenlyase and not hydrogenase. Our results indicate that a bacterium which possesses hydrogenlyase activity and not hydrogenase activity has yet to be found.

The activity of suspensions of *Bacillus lactis aerogenes* and *Proteus vulgaris* is completely inhibited with regard to the exchange reaction and the decomposition of formate by  $10^{-3}$  M cyanide, when the cyanide is added to the aerobic system before the suspension is tested for the reactions mentioned. It is apparent that we have not obtained any separation of hydrogenase from hydrogenlyase as exchange catalysts.

*Effect of Urethane on Methylene Blue Reduction*—Heavy suspensions of *Proteus vulgaris* fail to reduce methylene blue under hydrogen when the system is made 2 per cent with urethane, which is known to inhibit dehydrogenases. The exchange reaction is not inhibited by this concentration of urethane. It seems likely, therefore, that the reduction of methylene blue by the hydrogenase system is brought about by the addition of the gaseous hydrogen with the intervention of another enzyme system, from which the hydrogen ion and electron pair, necessary for the reduction of methylene blue, are contributed. We distinguish here between the effect of fumarate on the restoration of exchange activity to oxygen-inactivated suspensions of *P. vulgaris*, and its effect on the reduction of methylene blue in the presence of *P. vulgaris* and hydrogen. In the first case, the fumarate probably supplies the hydrogen necessary to reduce the enzyme responsible for the activation of molecular hydrogen. In the second case, fumarate acts as an intermediate through which hydrogen is transferred to methylene blue. That these two cases are distinguishable is evident from the fact that those suspensions which fail to reduce methylene blue in the absence of fumarate are nevertheless active with regard to the exchange reaction.

### Kinetics

*Effects of Bacterial Concentration and Temperature*—If the concentration of a bacterial suspension is increased, a point is reached at which the rate of the exchange reaction becomes independent of the bacterial concentration (see Fig. 7). The rate-determining step for the exchange reaction is then the rate at which the reacting gas diffuses to the catalyst. This has been confirmed by establishing the order of the exchange reaction and by determining the temperature coefficient of the exchange reaction under this condition. When diffusion of the reacting gases to the catalyst is rate-determining, the rate depends on the square root of the absolute temperature, and the kinetics of the reaction are of the first order. That the exchange reaction at high bacterial concentrations follows these conditions

is shown in Fig. 8, Curve A, and Fig. 9. Fig. 8, Curve A, shows the data from such an experiment to follow a first order equation,  $D = D_0 e^{-kt}$ , since  $\log D_0/D$  is linear with time. In Fig. 9 the square root of the absolute temperature is plotted against the rate constant; there is a linear relationship. It is therefore necessary, in order to determine rates other than that

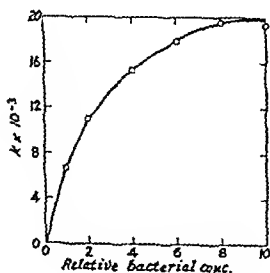


FIG. 7

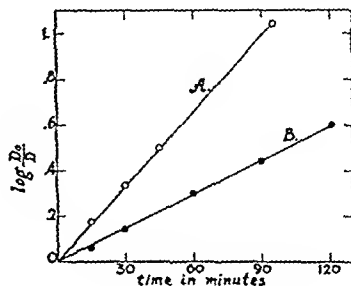


FIG. 8

Fig. 7. Effect of bacterial concentration on the rate of the exchange reaction. Fig. 8. The first order nature of the exchange reaction. Curve A, data showing that the first order equation  $D = D_0 e^{-kt}$  is followed; Curve B, that of Arrhenius.

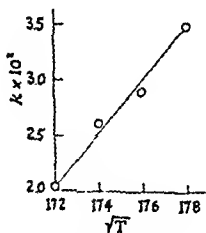


FIG. 9

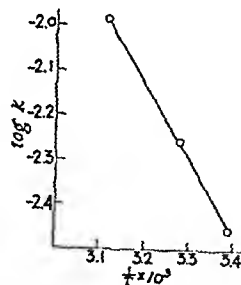


FIG. 10

Fig. 9. The effect of temperature on the rate of the exchange reaction when diffusion is rate-determining.

Fig. 10. The effect of temperature on the exchange reaction at low bacterial concentration.

of diffusion, to reduce the concentration of bacteria until another factor becomes rate-determining. When this adjustment is made, the kinetics of the exchange reaction remain of the first order, but the rate constants and the temperatures are related according to the equation of Arrhenius,  $k = A \cdot e^{-E/RT}$ . This is shown in Fig. 8, Curve B, and Fig. 10. The energy

of activation, calculated from the slope of the straight line obtained in Fig. 10, is 7.7 kilocalories.

The kinetics of the exchange reaction between a large excess of heavy water and hydrogen gas (with or without deuterium initially present) may be accurately represented by the following scheme.

Consider the removal of the volume element,  $dv$ , from the total gas volume,  $V$ , to the catalyst. Let a fraction,  $\beta$ , of this volume element come to equilibrium with the water on the catalyst. The concentrations of  $H_2$ , HD, and  $D_2$  in the volume,  $\beta dv$ , will be the equilibrium concentrations,  $(H_2)_e$ ,  $(HD)_e$ , and  $(D_2)_e$ , the concentrations being expressed in per cent. The volume element,  $dv$ , is returned to the bulk of the gas, with which it now becomes mixed. If  $X_e$  is the equilibrium concentration of any one of the gas species,  $H_2$ , HD, or  $D_2$ , and if  $X_t$  is the concentration of that species at time  $t$ , then the concentration of  $X$  will, after the time interval,  $dt$ , be

$$(6) \quad V X_{t+dt} = V X_t - X_t dv + X_e \beta dv + (1 - \beta) X_t dv$$

$$(7) \quad V dX = (X_e - X_t) \beta dv$$

If the rate at which the volume element,  $dv$ , is circulated through the catalyst, is constant, *i.e.* if

$$(8) \quad dv = k dt$$

then

$$(9) \quad V dX = (X_e - X_t) \beta k dt$$

$$(10) \quad \frac{dX}{(X_e - X_t)} = \beta k / V dt$$

If  $\beta$  and  $V$  are constant,

$$(11) \quad \beta k / V = 2.3/t \log \frac{(X_e - X_0)}{(X_e - X_t)} = K'$$

where  $X_0$  is the concentration of  $X$  when  $t$  is zero. In Fig. 11 are plotted the data obtained from a typical experiment in which a large excess of heavy water was shaken with normal hydrogen. The linear nature of the curves obtained by substituting concentrations of  $H_2$ , HD, and  $D_2$  in Equation 11 is good. If  $\beta$  is unity, that is, if all of the volume element,  $dv$ , is completely equilibrated with the water, then for the reaction between a large excess of normal water and heavy hydrogen, Equation 11 reduces to

$$(12) \quad K'' = 2.3/t \log X_0/X_t$$

for HD and  $D_2$  and to

$$(13) \quad K''_{H_2} = 2.3/t \log (100 - X_0)/(100 - X_t)$$

for  $H_2$ , since  $(HD)_0$  and  $(D_2)_0$  may be set equal to zero, and  $(H_2)_0$  to 100 per cent. Equation 12 should then describe the decrease in HD during the exchange reaction, and the decrease in  $D_2$ , and Equation 13 should describe the increase of  $H_2$ . In Table II are shown the concentrations of  $H_2$ , HD, and  $D_2$  and atom per cent D at various times, during an experiment in which heavy hydrogen gas at 470 mm. was shaken with a large excess of

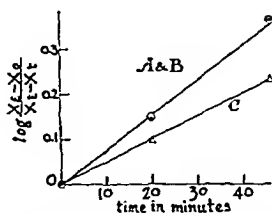


FIG. 11. Kinetics of the exchange reaction between a large excess of heavy water and normal hydrogen. In Curve A (●),  $X = H_2$ ; in Curve B (○),  $X = HD$ ; in Curve C (△),  $X = D_2$ .

TABLE II  
Effect of Pressure on Exchange Reaction

Pressure	Time	D	$H_2$	HD	$D_2$
mm.	min.	atom per cent	per cent	per cent	per cent
470	0	17.2	80.8	3.88	15.3
	15	15.0	83.0	4.13	12.9
	30	12.4	85.5	4.34	10.2
	60	8.60	89.3	4.24	6.48
	90	6.21	93.0	3.53	4.44
	121	4.34	94.1	3.05	2.81
30	0	17.2	80.8	3.88	15.3
	15	12.8	85.8	2.80	11.4
	30	9.51	89.2	2.54	8.23
	45	6.45	92.5	2.16	5.36
	60	4.66	94.5	1.77	3.77
	75	3.20	96.1	1.37	2.52

normal water. It will be seen that there is a period during which the concentration of HD in the gas increases. The assumption that equilibration between the entire volume of the gas brought to the catalyst and the water is complete is therefore incorrect, since if this were so, the concentration of HD would steadily decline.

In Table II is shown also the change in the concentration of the hydrogens with time, when the pressure of the gas is 30 mm. In Fig. 12 the values of  $\log (HD)_0 / (HD)_t$  obtained from Table II are plotted against

time. The data fit Equation 12 fairly well. It therefore appears that only at low pressures does the quantity,  $\beta$ , approach unity. In Fig. 13 is plotted the decrease in atom per cent D with time at the two pressures, 30 and 470 mm. It will be observed that the rate of the exchange reaction is faster at the low pressure.

The increase and subsequent decrease of IID at 470 mm. in the experiment recorded in Table II may be explained as follows: There exist two

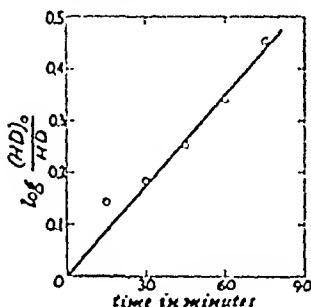


FIG. 12

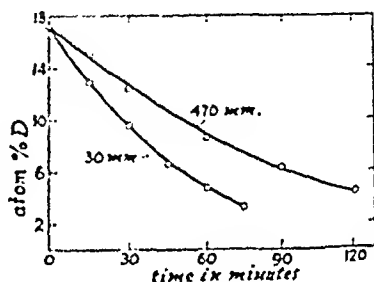


FIG. 13

FIG. 12. The first order nature of the disappearance of HD during the exchange<sup>c</sup> reaction between a large excess of normal water and heavy hydrogen when  $P = 30$  mm. of Hg.

FIG. 13. The rate of the exchange reaction between a large excess of normal water and heavy hydrogen for  $P = 30$  mm. of Hg and  $P = 470$  mm. of Hg.

possibilities to account for the formation of IID in this experiment: (1) direct reaction between  $\text{H}_2$  and  $\text{D}_2$  to give  $2\text{IID}$ , and (2) by the reaction



The almost complete disappearance of HD from the gas phase is then brought about by the reaction



If, at high pressure, the reaction of Equation 14 is not followed directly by that of Equation 15, that is, if the molecule of HD formed in Equation 14 does not make a collision with unbound enzyme, required for the completion of the reaction of Equation 15, HD appears in the gas phase. As the concentration of  $\text{D}_2$  decreases, the formation of HD also decreases, and HD disappears according to Equation 15.

It is also possible that reaction between  $\text{D}_2$  and  $\text{H}_2$  will also give rise to IID. In this case also, as the concentration of  $\text{D}_2$  decreases, the HD formed disappears according to Equation 15.

At low pressures the rate of the exchange reaction is faster than at high pressures. The possibility therefore of both reactions (Equations 14 and

15) going to completion is increased, since the concentration of enzyme not bound to the hydrogens is greater than at high pressures.

Because the exchange reaction proceeds faster at low pressure than at high, the concentration of HD for the two experiments, is, for purposes of comparison, better represented as a function of the total atomic concentration of deuterium, than as a function of time. It is then possible to compare HD concentrations at the two pressures point for point. This is

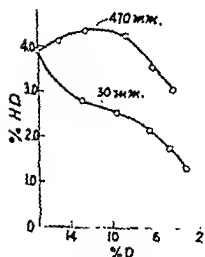


FIG. 14

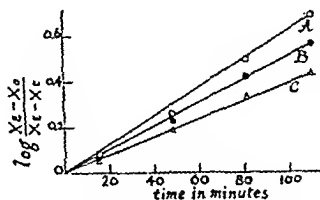


FIG. 15

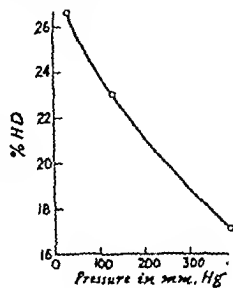


FIG. 16

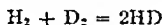
FIG. 14. The effect of pressure on the formation of HD during the exchange reaction between a large excess of normal water and heavy hydrogen.

FIG. 15. The kinetics of the exchange reaction when a large excess of heavy water is shaken against heavy hydrogen containing the equilibrium concentration of D. In Curve A,  $X = H_2$ ; in Curve B,  $X = HD$ ; in Curve C,  $X = D_2$ .

FIG. 16. The kinetics of the exchange reaction for HD when a large excess of heavy water is shaken against heavy hydrogen containing the equilibrium concentration of D.

shown in Fig. 14, in which the increase and decrease of HD at 470 mm. are evident. In order to determine the effect of pressure on the reaction

(16)



the following experiment was carried out. When a bacterial suspension in a large amount of heavy water is shaken with deuterium gas containing that atomic concentration of deuterium which is in equilibrium with the water, the concentration of deuterium atoms in the gas and in the water remains constant. If, however, the concentrations of  $H_2$ , HD, and  $D_2$  in this gas mixture are not in equilibrium, there is a shift in concentration of the hydrogens until they are ultimately present in equilibrium concentrations. We have found that the data obtained from such an experiment fit Equation 11. This is shown in Fig. 15.

When the effect of pressure on the rate of formation of HD is studied, it is found that the rate of formation of HD increases with low pressures,



just as does the exchange rate in the experiment previously described. This is shown in Fig. 16 in which the concentration of HD formed in 63 minutes is plotted against pressure. If the formation of HD at high pressure were due to the reaction represented by Equation 16, the effect of a decrease in pressure would be to decrease the rate of formation of HD, since the rate of a bimolecular reaction is proportional to the product of the concentration of the reactants. The results shown in Fig. 16 indicate that the formation of HD takes place primarily through the reaction described in Equation 14.

#### SUMMARY

The results obtained from the experiments described may be summarized as follows:

1. The activity of the enzyme responsible for the exchange reaction between water and hydrogen is reversibly inhibited by oxygenation. There is evidence that this inactivation is caused by oxidation of a heavy metal in the enzyme. This and other findings suggest that the enzyme responsible for the exchange reaction is probably an iron porphyrin-protein complex.
2. The reactivation of the exchange reaction probably occurs by reduction of the oxidized enzyme by biological reducing agents.
3. The activities of two enzymes which may be responsible for the exchange reaction, hydrogenase and hydrogenlyase, were not separable.
4. The reduction of methylene blue by hydrogen may be inhibited by urethane, without effect on the exchange reaction.
5. The rate-determining step of the exchange reaction between water and hydrogen, catalyzed by low concentrations of *Proteus vulgaris*, is the rate of chemical reaction between water and hydrogen.
6. At high bacterial concentrations, the rate-determining step becomes the rate at which hydrogen diffuses to the enzyme.
7. The effect of increased pressure on the exchange reaction is to bring about a diminution in the concentration of unbound enzyme, and the rate decreases with increase in pressure.
8. The reaction  $H_2 + D_2 = 2HD$  does not take place to a significant extent.

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## A STUDY OF THE ACTION OF PANCREATIC AMYLASE. II

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A study of the influence of a number of reagents upon the action of pancreatic amylase (1) has shown that the primary amino groups of the enzyme protein molecule are essential to the activities of this amylase. The work reported here with nitrous acid extends and strengthens this finding and shows more conclusively that the phenolic tyrosine groups of the molecule are not concerned with its amylase activity.

Nitrous acid is especially well suited to such an investigation. It reacts with the free amino and with the free tyrosine phenolic groups of native proteins in certain specific ways (2) without disrupting the molecule as a whole (3), and these reactions may easily be followed (2-4) and are readily distinguished from one another (3, 5). The reactions with the primary and with the secondary amino groups are both of the third order, and both become second order in the presence of excess nitrous acid (5). The reaction of nitrous acid with the tyrosine phenolic groups of proteins, on the other hand, is of the second order and becomes first order in the presence of excess nitrous acid (3). Moreover, the reactions of nitrous acid with the primary and secondary amino groups of proteins are much more rapid than that with their tyrosine phenolic groups (5).

The final diazo compound which results from the action of nitrous acid upon the phenolic group of tyrosine has a yellow color. This makes it possible readily to follow the reaction by the measurement of the increase in the absorption of light. This is carried out at a wave-length of 411.5  $m\mu$  (3). Furthermore, it has been shown by Philpot and Small (3) that the presence of a peptide link involving the amino group of tyrosine makes no difference in the rate of the reaction of the tyrosine phenolic groups with nitrous acid. These authors found that the rate and order of the reaction for the loss of the activity of pepsin, upon treatment with nitrous acid under certain closely defined conditions, were exactly the same as those of the reaction of nitrous acid with free tyrosine or with  $\alpha$ -bromopropionyltyrosine, under the same conditions. These findings were used by Evans and his coworkers (6) in their work with the hormone which stimulates pigeon crop formation. They have also been helpful in the study and interpretation of the results obtained here for the influence of nitrous acid upon the activity of pancreatic amylase.

## EXPERIMENTAL

*Treatment with Nitrous Acid*—After a number of preliminary experiments, the following procedure was adopted for the treatment of the amylase with nitrous acid. Purified pancreatic amylase (7) was dissolved at 0° in an aqueous solution at pH 4.6 in the presence of 0.5 M acetate, 0.02 M phosphate, and 0.01 M sodium chloride. The phosphate and chloride were added to aid in the stability of the enzyme (8). An aliquot of this solution was diluted with an equal volume of cold water and held in an ice bath in the dark (7) as a control. Another aliquot was treated with an equal volume of 2 M sodium nitrite. This was kept in the ice bath in the dark and samples were withdrawn at the intervals noted, diluted, and adjusted to pH 7.1 for activity measurements. For this, equal volumes of these solutions reacted for 30 minutes at 40° with 1 per cent soluble potato starch at pH 7.1 (8), and the resulting reaction mixtures were measured for reducing value (9), which was calculated in terms of maltose. All activities were compared with that of the corresponding untreated control solution which had been held under exactly the same conditions but without addition of nitrite.

The rate and the order of the reaction for the loss of the saccharogenic activity of the amylase upon treatment with nitrous acid were determined and compared with those for the formation of the diazo compound from pure tyrosine under identical conditions by use of a Coleman double monochrometer spectrophotometer, model 10S. It was not possible to follow the rate of formation of the diazo compound in the enzyme solution itself, as this was slightly turbid and the treatment with the nitrous acid caused a precipitate to form which interfered with the absorption measurements.

*Results*

Typical results with the enzyme solutions and with solutions of pure tyrosine are given in Tables I and II.

A study of these data shows that the rate and the order of the inactivation reaction of the amylase are very different from those for the action of nitrous acid with the phenolic groups of tyrosine, under the same conditions. The rate of the inactivation of the amylase by nitrous acid is much more rapid than that of the reaction of nitrous acid with tyrosine, and is probably more nearly of the magnitude of the rate of the reaction between amino groups and nitrous acid (5).

Moreover, when the reciprocals of the fractions of the original amylase activity which remain at any time, listed in the last column of Table I, are plotted against the time of reaction, a straight line is obtained. This

TABLE I  
Inactivation of Pancreatic Amylase by Nitrous Acid

Reaction time	Maltose per 2 cc. reaction mixture	Saccharogenic activity	Fraction of original activity remaining	Reciprocals
min.	mg.	S	$\frac{s}{s_0}$	$\frac{1}{\frac{s}{s_0}}$
0				
2½	6.55	6550	1.000	1.00
5	5.78	5780	0.882	1.13
10	4.77	4770	0.728	1.37
15	4.00	4000	0.609	1.64
20	3.23	3230	0.493	2.03
25	2.89	2890	0.441	2.27
30	2.47	2470	0.377	2.65
30 (Control)	2.21	2210	0.337	2.97
	6.38	6380	0.974	1.03

$$k = \frac{d(s_0/s)}{dt(\text{minutes})} = 0.0657; \text{ half life} = 14.3 \text{ minutes.}$$

TABLE II  
Rate of Reaction of Nitrous Acid with Tyrosine

Reaction time	Per cent transmission at 411 mμ	Log 100 minus log per cent transmission	Fraction of tyrosine remaining*	Log of fraction of tyrosine remaining
min.				
0	100.0	0.0000	1.0000	0
50	100.0	0.0000	1.0000	0
90	97.7	0.0101	0.9849	-0.0066
150	90.6	0.0429	0.9369	-0.0283
240	80.4	0.0947	0.8607	-0.0652
390	67.2	0.1726	0.7461	-0.1272
490	60.7	0.2168	0.6811	-0.1668
730	48.0	0.3188	0.5311	-0.2748
790	45.6	0.3410	0.4985	-0.3023
1395	33.2	0.4789	0.2956	-0.5293
1590	30.9	0.5100	0.2499	-0.6022
1750	29.3	0.5331	0.2159	-0.6658
2045	26.7	0.5735	0.1565	-0.8055
2795	23.4	0.6308	0.07221	-1.1414
3230	22.3	0.6517	0.04148	-1.3822
4235	21.2	0.6737	0.00912	-2.0400
∞	20.9	0.6799	0.00000	

$$k_m = - \frac{d \log (T/T_0)}{dt (\text{minutes})} = 0.000413; \text{ half life} = 790 \text{ minutes.}$$

$$* 1 - \frac{(\log 100 - \log \% \text{ transmission})}{0.6799}.$$

shows that the inactivation of the amylase by nitrous acid is of the second order and that in this respect also it resembles the reaction of nitrous acid with amino groups (5). On the other hand, when the logarithms of the fractions of tyrosine which remain at any time, given in the last column of Table II, are plotted against the time of reaction, the straight line which results shows that the reaction of tyrosine with nitrous acid under the same conditions is of the first order for times up to 1750 minutes (3).

If the widely different rate constants and half life times, which are listed below the tables are also taken into consideration, it is obvious that the inactivation of this enzyme by nitrous acid cannot be due to a reaction with the tyrosine of the protein but is probably caused by reaction with its free amino groups. This latter conclusion is strengthened by the finding, reported elsewhere (1), of a close correlation between the loss of the activity of the amylase and the loss of its amino nitrogen upon acetylation with ketene.

#### SUMMARY

The influence of nitrous acid upon the saccharogenic activity of pancreatic amylase has been studied.

The results show that the rate and the order of the inactivation reaction of the amylase are very different from those for the action of nitrous acid with the phenolic groups of tyrosine. They give additional evidence that the activity of this enzyme is intimately connected with the free primary amino groups of the intact protein molecule.

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# THE ULTRAVIOLET ABSORPTION OF VITAMIN K<sub>1</sub> AND THE EFFECT OF LIGHT ON THE VITAMIN

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The relationship between chemical structure and ultraviolet absorption spectra is nowhere illustrated better than in the study of vitamin K and related compounds. One generalization which we presented in a previous publication (1), namely that the vitamin K absorption curve presents a summation of the benzenoid and the quinoid components of the disubstituted naphthoquinone molecule, recently has been extended by Morton and Earlam (2) to the anthraquinone series.

We now have made a more complete study of the vitamin K<sub>1</sub> absorption curve, using not only the vitamin isolated from natural sources, but also several specimens of the synthetic product. The main objects of the work were to check the identity of the natural and synthetic products and to determine whether the absorption curve, and especially the absorption coefficient at  $\lambda$  249  $\mu$ , could be used as a measure of purity of the vitamin. It was hoped also that the results of this detailed study might clear up the controversy between Karrer (3, 4) and Doisy (5, 6) regarding the absorption coefficients of the pure vitamin. Obviously, in order to contribute to this question it was necessary to reinvestigate the complete structure of the absorption curve and to study the influence of light and other factors such as the presence of acetic acid which sometimes is added as a stabilizer.

## *Apparatus and Materials*

The samples of vitamin K<sub>1</sub>, both natural and synthetic, used in this investigation were prepared by Doisy and his associates at the St. Louis University School of Medicine. They were examined with a Bausch and Lomb medium quartz spectrograph and ultraviolet sector photometer, with a Hilger No. H-698 hydrogen discharge tube as the source of continuous ultraviolet light.

The hexane used as the solvent in this study was the Eastman Practical grade, which was purified and redistilled. The purification process consisted of five to ten shakings with 10 per cent fuming sulfuric acid, two washings with 10 per cent Na<sub>2</sub>CO<sub>3</sub> solution, prolonged shaking with 5 per cent KMnO<sub>4</sub>-10 per cent Na<sub>2</sub>CO<sub>3</sub> solution, fifteen to twenty washings with distilled water, drying for 24 hours over calcium oxide, and distillation



in hexane solution, in the absence of acetic acid, which was exposed to  $\lambda$  365.5 and 366.3  $m\mu$  lines of mercury radiation, readings being taken at 0, 15, 30, 45, 60, 90, 135, 195, and 255 minutes, respectively. It will be noted that the exposure produces a gradual lowering of the maxima at  $\lambda$  239, 243, 249, 260, and 269  $m\mu$ , and a less pronounced decrease in the maximum at  $\lambda$  325  $m\mu$ , indicating a gradual decomposition of the vitamin.

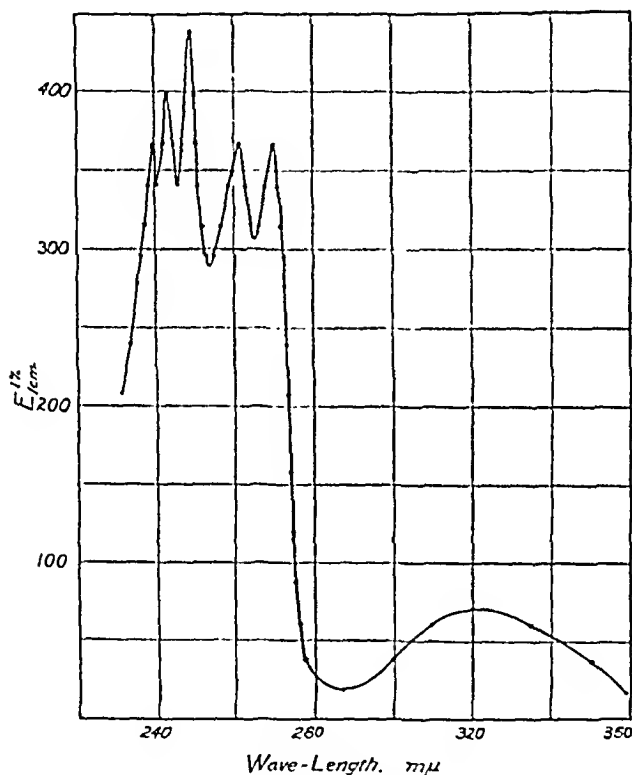


FIG. 2. Absorption curve of synthetic vitamin K<sub>1</sub> in hexane

Fig. 4 shows a similar set of curves for a sample of synthetic vitamin K<sub>1</sub>, containing a small amount of acetic acid, determined under identical conditions as those illustrated in Fig. 3. The curves are essentially the same except for a slight stabilizing effect of the acetic acid during the first few exposures.

In both of the above cases, the initial effect is most pronounced on the maxima at  $\lambda$  260 and 269  $m\mu$ , which we previously have shown to be associated with the quinone structure ((1) p. 350). It follows, therefore, that the point of attack is through the quinone grouping. In Figs. 3

and 4 it is interesting to note that the nine curves intersect at approximately  $\lambda$  277  $m\mu$ , and that less definite isoextinction coefficient points occur at  $\lambda$  230  $m\mu$  and  $\lambda$  305  $m\mu$ .

No attempt was made to correct for the effect of exposure of the sample to ultraviolet light from the hydrogen discharge tube during the exposure of the plate, because a series of runs made for the purpose showed that the effect of this light was negligible.

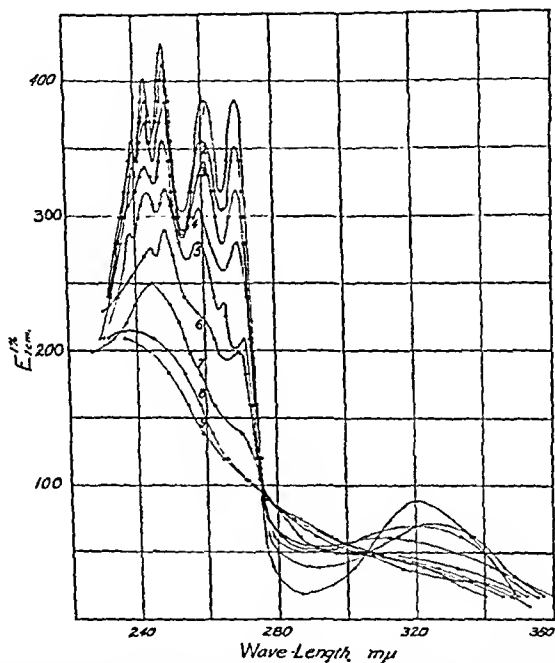


FIG. 3. Absorption curves showing the effect of ultraviolet radiations on natural vitamin  $K_1$  in hexane. The readings for the curves, in numerical order as indicated, were taken at 0, 15, 30, 45, 60, 90, 135, 195, and 255 minutes.

MacCorquodale, Binkley, *et al.* (7) report that vitamin  $K_2$  is unstable when exposed to light, and curves were presented in our previous article showing the deterioration of the absorption curves of samples of vitamins  $K_1$  and  $K_2$  when exposed to diffuse daylight. In order to determine, if possible, the wave-lengths or wave-length of light causing this decomposition, samples of vitamin  $K_1$  were exposed to various wave-length regions from infra-red to ultraviolet. The results are shown in Table I.

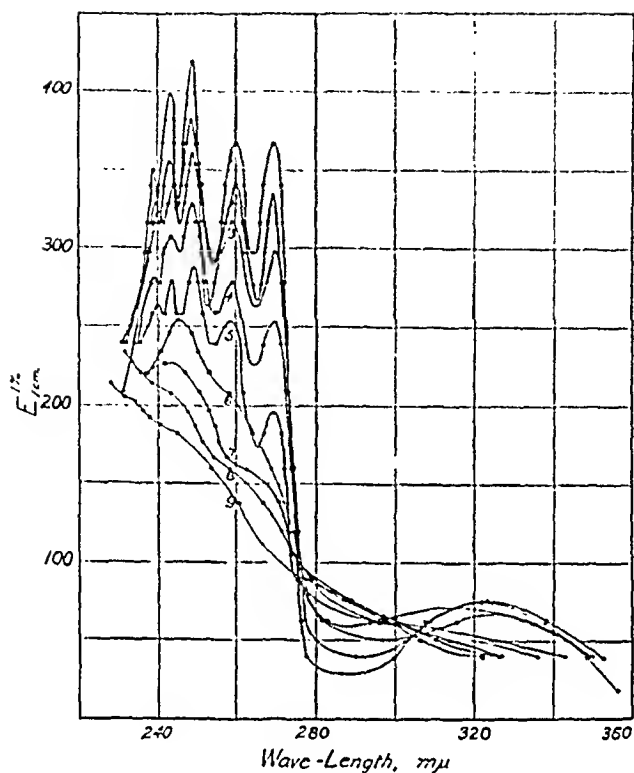


FIG. 4. Absorption curves showing the effect of ultraviolet radiations on synthetic vitamin K<sub>1</sub> in hexane. The readings for the curves, in numerical order as indicated, were taken at 0, 15, 30, 45, 60, 90, 135, 195, and 255 minutes.

TABLE I  
*Effect of Various Radiations on Vitamin K<sub>1</sub>*

Source of radiation	Filters used	Time	Effect
Nernst glower	None	3 hrs.	Slight decomposition
" "	Zeiss No. R-30	3 "	No effect
Tungsten filament lamp	Wratten A	45 min.	" "
" " "	" B	45 "	" "
" " "	" C	45 "	" "
Hg arc	Cenco ultraviolet-transmitting + Cenco infra-red-absorbing	15 "	Decomposition
Diffuse daylight	None	2 hrs.	"

These data show that light radiations between  $\lambda$  400 and 800 m $\mu$  have no appreciable effect on the vitamin. The slight decomposition shown in the case of the Nernst glower with no filter was probably due to the ultra-

violet light present in the radiation from the incandescent filament of the glower. Thus, the decomposition reported earlier as being due to the effect of visible light probably was due to the small amount of ultraviolet light present in diffuse daylight.

We reported previously that vitamin  $K_1$  was unstable in hexane solution in the dark. However, using specially purified hexane, we now can report that vitamin  $K_1$  in dilute hexane solution is stable for periods up to 5 months when stored in the dark at room temperature.

#### DISCUSSION

Since Karrer and Doisy first began publication on vitamin  $K_1$ , there has been a discrepancy between the two laboratories concerning the correct value for  $E_{1\text{cm}}^{1\%}$  at  $\lambda$  249  $m\mu$ . In their first publication Dam *et al.* (8) gave a value of 280 and McKee *et al.* (5) a value of 385. In subsequent publications Karrer (3, 4) has claimed that his vitamin preparation was pure and that 280 was the correct value for the extinction coefficient. In an effort to discern the cause of the discrepancy, we have made numerous measurements on both natural and synthetic vitamin  $K_1$  samples which were prepared and supplied to us by Dr. Doisy. We find that  $E_{1\text{cm}}^{1\%}$  at  $\lambda$  249  $m\mu$  is  $435 \pm 5$  ( $\log E_m = 4.29$ ). This value is in good agreement with values reported by D. M. Bowen (9) ( $\log E_m = 4.24$  to 4.27) (in alcoholic solution) and T. J. Webb (9) ( $\log E_m = 4.26$ ) (alcoholic solution). We believe that this is the correct value for either pure natural or synthetic vitamin  $K_1$  in hexane solution.

Of particular interest in connection with the controversy between the two laboratories is the fact that both groups agree on the values for  $E_{1\text{cm}}^{1\%}$  for vitamin  $K_2$  (1, 4) and for the diacetate of dihydrovitamin  $K_1$  ( $\log E_m = 4.93$ ) (1, 4).

The close agreement of our results with those of other laboratories leaves Karrer's low values for  $E_{1\text{cm}}^{1\%}$  for vitamin  $K_1$  unexplained. From a structural point of view the chief difference between vitamin  $K_1$  and vitamin  $K_2$  is the size of the side chain in the 3 position. Since the side chain in each case is aliphatic and contains no conjugated double bonds, the absorption spectra for the two compounds would be expected to be quite similar. If the absorption is due to the naphthoquinone portion of the molecule and is not influenced by the size of the aliphatic side chain in the 3 position, the  $E_{1\text{cm}}^{1\%}$  values for the two vitamins should be inversely proportional to their molecular weights and the  $\log E_m$  values should be equal. The same reasoning holds true for the diacetates of vitamins  $K_1$  and  $K_2$ . Actually this is the case. The  $\log E_m$  values for vitamins  $K_1$  and  $K_2$  are 4.27 and 4.29 respectively, and the values for the corresponding diacetates are 4.93 and 4.93. It is significant that the molar extinction coefficients obtained for vitamins  $K_1$  and  $K_2$  agree well with values obtained by Tishler *et al.* (9)

for three crystalline 2,3-dialkyl-1,4-naphthoquinones. These quantitative relationships are good evidence in support of the correctness of our values.

A comparison of the absorption curves previously published by us (1), as well as those now presented, with that illustrated in the article by Dam *et al.* (8) suggests that there might be a proportionate discrepancy between the heights of the respective extinction coefficients at  $\lambda$  249 m $\mu$  and  $\lambda$  325 m $\mu$ . As a matter of fact, the discrepancy is not serious. Karrer's curve was plotted as  $\log E_{1\text{ cm}}^{1\%}$  versus wave-length, which tends to enhance the  $\lambda$  325 m $\mu$  maximum, and was compared in this form with our curve which was plotted as  $E_{1\text{ cm}}^{1\%}$  versus wave-length. This latter method shows better the fine structure in the region  $\lambda$  239 m $\mu$  to  $\lambda$  270 m $\mu$ , but gives a less pronounced maximum at  $\lambda$  325 m $\mu$ .

An examination of the absorption curves showing the effect of ultraviolet light on solutions of vitamin K<sub>1</sub> gives a certain amount of information as to the actual chemical change involved. We have stated in the experimental part of this paper that the point of attack probably is through the quinone grouping, and may add that the  $\lambda$  325 m $\mu$  maximum which we previously associated with the ring structure changes more slowly than the rest of the curve.

2-Methyl-1,4-naphthoquinone upon exposure to light for long periods of time is decolorized and forms a polymer of known structure (10). It is possible that a similar reaction occurs when vitamin K<sub>1</sub> is exposed to light.

#### SUMMARY

1. A more careful examination of the absorption curve of vitamin K<sub>1</sub> shows the presence of a new maximum at  $\lambda$  239 m $\mu$ .
2. The  $E_{1\text{ cm}}^{1\%}$  of pure vitamin K<sub>1</sub> at  $\lambda$  249 m $\mu$  is  $435 \pm 5$ .
3. Vitamin K<sub>1</sub> in hexane solution is stable upon standing in the dark at room temperature for as long as 5 months.
4. Vitamin K<sub>1</sub> in hexane solution is decomposed rapidly by the action of ultraviolet light, while visible and infra-red radiations have no effect. The point of attack probably is through the quinone group.
5. The presence of acetic acid has no noticeable effect on the absorption curve.
6. On the basis of the absorption values reported by Dam, Geiger, Glavind, Karrer, Karrer, Rothschild, and Salomon, their product appears to have been 60 to 80 per cent pure.

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# ENZYMATIC FIXATION OF CARBON DIOXIDE IN OXALACETATE\*

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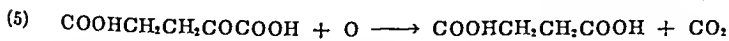
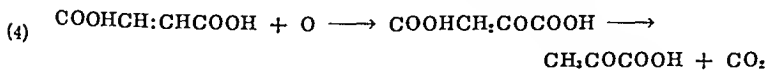
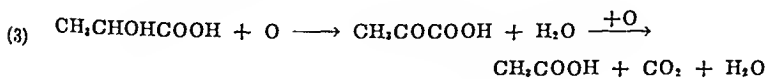
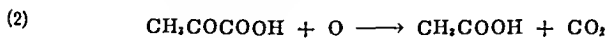
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According to Wood and Werkman (1-3), carbon dioxide enters the metabolic processes in heterotrophic organisms in conformity with the following empirical equation.



Krampitz and Werkman (4) have obtained an enzyme from *Micrococcus lysodeikticus*, which decarboxylates oxalacetate to pyruvate, and have indicated their belief that the same enzyme is responsible for fixation of  $\text{CO}_2$ . Their attempts to demonstrate the carboxylation of pyruvate to oxalacetate were unsuccessful, but it was recognized that the possibility of carboxylation was not excluded, since the equilibrium of the reaction may be far to the side of decarboxylation and the quantities of oxalacetate formed may be too small for detection. Alternatively, as stated by Wood *et al.* (3), a phosphorylated form of pyruvate, rather than pyruvate as such, may be the active metabolic compound in the carboxylation reaction.

The purpose of this communication is to report the results of experiments on enzymatic and non-enzymatic exchanges of  $\text{C}^{13}\text{O}_2$  with the carboxyl groups of oxalacetate in Reaction 1 and with the carboxyl groups of other acids in Reactions 2 to 5.



The authors consider any exchange reaction with carbon dioxide involving the formation of a carbon-to-carbon linkage to be a fixation reac-

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tion. Wood and Werkman (1) have proposed that the usual mechanism of heterotrophic fixation of  $\text{CO}_2$  occurs by  $\text{C}_3$  and  $\text{C}_1$  addition. Any exchange of carbon dioxide with any other than a 4-carbon compound would indicate the existing proposal to be incomplete in that other mechanisms of fixation have been neglected.

Krebs and Eggleston (5), from indirect evidence with pigeon liver experiments, have considered Reaction 1 as adequately demonstrated. It is obvious that the acceleration of pyruvate dissimilation or the obtaining of larger quantities of succinate in the presence of bicarbonate is not rigid proof of the reaction.

Evans (6) employed yeast carboxylase and found no exchange of  $\text{C}^{14}\text{O}_2$  with pyruvate. Ruben and Kamen (7) have considered this same reaction to be irreversible; however, Carson, Ruben, Kamen, and Foster (8) have recently stated that a very small amount of  $\text{C}^{14}\text{O}_2$  is utilized by carboxylase preparations in the presence of acetaldehyde and pyruvic acid. Other than these reports, there is no information regarding exchange of carbon dioxide in reactions involving cleavage of a carbon-to-carbon linkage.

### Methods

In general the reactions were conducted in the presence of  $\text{NaHC}^{13}\text{O}_3$  until approximately one-half of the original substrate remained. The residual substrate was degraded and the  $\text{C}^{13}$  content of the fragments determined on the mass spectrometer (Nier (9)).

Enzyme preparations of *Micrococcus lysodeikticus* were prepared according to Kramnitz and Werkman (4). Acetone-treated cells decarboxylate oxalacetate to pyruvate and  $\text{CO}_2$  and oxidatively decarboxylate pyruvate to acetate and  $\text{CO}_2$ . Cells deficient in  $\text{Mg}^{++}$  and cocarboxylase do not decarboxylate either oxalacetate or pyruvate; however, the addition of  $\text{Mg}^{++}$  restores the ability to decarboxylate oxalacetate, whereas the addition of cocarboxylase and  $\text{Mg}^{++}$  is required for the decarboxylation of pyruvate.

$\text{NaHC}^{13}\text{O}_3$  was prepared from  $\text{C}^{13}\text{O}_2$  obtained from methane whose  $\text{C}^{13}$  content had been increased in a thermal diffusion column according to Nier and Bardeen (10).

The exchange reactions were conducted in 125 ml. Erlenmeyer flasks with two side arms, which were attached to Warburg-Barcroft manometers. 300 mg. of the acetone preparation were used in each flask. The concentration of the oxalacetic acid was 0.053 M and that of all other acids was 0.026 M. Each acid was adjusted to pH 7.0 before addition. The mixture was buffered with 0.021 M phosphate, pH 6.6, and 0.05 M  $\text{NaHC}^{13}\text{O}_3$ . Appropriate concentrations of the two buffers were placed in the side arms of the flask and mixed with the enzyme preparations and substrate after

temperature equilibrium (30.4°) had been reached. The approximate pH of the resulting mixture was 7.2, and the volume was 30 ml. Owing to the instability of oxalacetate to acid reactions and the length of time required for analyses, the concentration of this acid was doubled so that substantial quantities remained for analysis. At the conclusion of the reaction (cf. Tables I and II) the enzyme preparation was rapidly centrifuged off and analyses were made on the supernatant liquid.

Because of the instability of oxalacetate to acidification and heat, the residual  $C^{13}O_2$  was not removed by boiling and aeration. Preliminary experiments conducted at room temperature with the phosphate-bicarbonate buffer mixture and oxalacetate proved that on acidification to Congo red the  $CO_2$  is quantitatively removed from solution by aeration through a sintered glass disk for 15 minutes.

In the experiments the solution was acidified and aerated for 15 minutes, then 0.75 mm of  $NaHC^{12}O_3$  was added and the aeration was repeated. The use of a  $C^{12}O_2$  rinse insured that no  $C^{13}O_2$  remained to interfere subsequently with the isotope determination of the carboxyl carbons. The  $C^{13}$  content of the  $CO_2$  from this normal bicarbonate rinse was determined and compared with the  $C^{13}$  concentration of the acid analyzed. The values in parentheses in Table I give the  $C^{13}$  content of the rinse.

The aeration was conducted in a 20 × 5 cm. Pyrex tube. Arrangements were made for the addition of the necessary reagents during the course of aeration. The  $CO_2$  was collected in 20 ml. of 1.5 N carbonate-free NaOH in a bead tower. In all experiments except those which first required ether extraction, the mixture was centrifuged and the supernatant liquid was used.

The oxalacetate in the resulting  $CO_2$ -free medium was degraded by the two following methods.

*Aniline-Citrate Method (Edson (11))*—In this method  $CO_2$  originates from the carboxyl adjacent to the methylene group of oxalacetate. This carbon dioxide was collected and its  $C^{13}$  content determined. The other carboxyl carbon is linked with aniline as pyruvanilide.

*Acid and Heat Decarboxylation*—When oxalacetate is made acid to Congo red with  $H_2SO_4$  and boiled for 30 minutes, it is quantitatively decarboxylated to pyruvate and carbon dioxide. This  $CO_2$  originates from the carboxyl group adjacent to the methylene group. The advantage of this method is that the resulting pyruvate can be oxidized with ceric sulfate at room temperature to acetic acid and carbon dioxide (Fromageot and Desnuelle (12)). The  $CO_2$  originates from the carboxyl group of pyruvate. In this way carbon atoms in the two carboxyl groups of the oxalacetate can be separated for the  $C^{13}$  determination.

In the experiment in which  $\alpha$ -ketoglutarate was the substrate (Reaction

5) the residual  $\alpha$ -ketoglutarate was degraded with  $\text{KMnO}_4$  (Wood *et al.* (3)). The products of this oxidation are succinate and carbon dioxide. The carbon dioxide which originates from the carboxyl adjacent to the carbonyl group was collected and the  $\text{C}^{13}$  content determined.

When lactate was used as the substrate (Reaction 3), the residual lactate and the oxidation product, pyruvate, were separated by continuous ether extraction after addition of bisulfite. The ether extract contained the lactic acid. The pyruvate-bisulfite complex in the residue was decomposed by boiling and the extraction repeated to recover the pyruvic acid. The carboxyl group of the lactic acid was decarboxylated by  $\text{KMnO}_4$  oxidation (Friedemann and Kendall (13)), and that of the pyruvic acid by ceric sulfate oxidation.

### EXPERIMENTAL

Experiments shown in Table I, Column 3, were conducted with oxalacetate and  $\text{NaHC}^{13}\text{O}_3$  in the absence of enzyme. The extent of exchange of carbon dioxide occurring during this spontaneous decarboxylation was measured as a standard for comparison with the enzymatic exchange. The results show that there may be a very slow rate of exchange between carbon dioxide and the carboxyl group adjacent to the methylene group. However, the  $\text{C}^{13}$  percentage, 1.13, with the aniline-citrate method and 1.12 with the acid-heat method, is only slightly above the normal of 1.09 and lies almost within the limits of error of the mass spectrometer ( $\pm 2$  per cent). The  $\text{C}^{13}$  value of the rinse was nearly normal, showing that the  $\text{C}^{13}\text{O}_2$  was practically completely removed. It should be emphasized that the time of these experiments was much longer (210 minutes) than that of the experiments with the enzyme preparation so as to allow approximately the same amount of decarboxylation. The  $\text{C}^{13}$  content of the carboxyl group adjacent to the carbonyl group is normal.

Swendseid *et al.* (14) have shown that the natural abundance of  $\text{C}^{13}$  in animal tissues averages lower than that in normal  $\text{NaHCO}_3$ . Because of this variation there is some question as to the  $\text{C}^{13}$  value which should be used as the standard in judging whether a compound contains fixed  $\text{C}^{13}\text{O}_2$ . Therefore the  $\text{C}^{13}$  content was measured in oxalacetate obtained from experiments in which there was enzymatic exchange with normal  $\text{NaHCO}_3$  and was found to be (Table I, Column 4) comparable to that in normal  $\text{NaHCO}_3$ ; *i.e.*, 1.09 per cent. Apparently there was no detectable differentiation of the isotopes of carbon, as seemed to have occurred in the animal experiments of Swendseid *et al.* All of the  $\text{C}^{13}$  values given are the average of several experiments. The results in Column 4 were used as the standard in judging whether fixation of  $\text{C}^{13}$  had occurred in the oxalacetate.

Experiments conducted with the acetone-enzyme preparation, oxalacetate, and phosphate-bicarbonate buffer mixture show a very significant

carbon dioxide exchange. The carboxyl group adjacent to the methylene group contained 1.29 per cent  $C^{13}$  (acid-heat method, Table I, Column 5), a value 18.3 per cent above the normal  $C^{13}$  complement of 1.09 per cent. By the aniline-citrate method the value was found to be 1.23 per cent. The  $C^{13}$  concentration of the  $NaHC^{13}O_3$  rinse was 1.12 per cent, showing definitely that the residual  $NaHC^{13}O_3$  was not interfering with the carboxyl carbon determination. These results show that there is a rapid rate of exchange in the presence of the enzyme in contrast to the insignificant exchange in the absence of the enzyme.

TABLE I

*Exchange of Heavy Carbon Dioxide in Carboxyl Groups of Oxalacetate during Enzymatic and Non-Enzymatic Decarboxylation*

The  $C^{13}$  concentration of the original bicarbonate was 9 per cent. The bold-faced figures represent  $C^{13}$  values when exchange has taken place. The values enclosed in parentheses are the  $C^{13}$  contents of the rinse. All readings are measured in per cent  $C^{13}$ .

Group to which carboxyl group is adjacent	Method of degradation	Spontaneous decarboxylation, 210 min., $C^{13}O_2$ added	Enzymatic decarboxylation			
			Acetone preparation, 20 min., $C^{13}O_2$ added	Acetone preparation, 20 min., $C^{13}O_2$ added	Mg <sup>++</sup> -deficient preparation, 60 min., $C^{13}O_2$ added	Deficient preparation + Mg <sup>++</sup> , 20 min., $C^{13}O_2$ added
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Methylene	Aniline-citrate	1.13 (1.12)	1.10	1.23 (1.13)	1.16 (1.09)	1.26 (1.11)
"	Acid-heat	1.12 (1.11)	1.10	1.29 (1.12)	1.16 (1.12)	1.26 (1.08)
Carbonyl	Ceric sulfate	1.11	1.09	1.11	1.08	1.11

The  $C^{13}$  concentration of the carboxyl group adjacent to the carbonyl group was 1.11 per cent, indicating that no exchange had taken place in this position.

As reported by Krampitz and Werkman (4)  $Mg^{++}$  but neither thiamine nor cocarboxylase was required for the decarboxylation of oxalacetate. The  $Mg^{++}$ -deficient preparation was employed with oxalacetate and the buffer mixture (Table I, Column 6). The  $C^{13}$  concentration of the carboxyl group adjacent to the methylene group was 1.16 per cent, as determined by both the aniline-citrate and acid-heat methods of decarboxylation. This small fixation of  $C^{13}$  indicates that there was a slow exchange which may have been due to incomplete removal of the magnesium ions. The carboxyl group adjacent to the carbonyl group had a  $C^{13}$  concentration of 1.08 per cent, indicating that no exchange had taken place.

When  $Mg^{++}$  was supplied to the deficient preparation, exchange took

place as evidenced by the 1.26 per cent of  $C^{13}$  in the carboxyl group adjacent to the methylene group (Table I, Column 7). This concentration was 15.6 per cent above the normal complement of  $C^{13}$ , by both methods of analysis. The carboxyl adjacent to the carbonyl group had a  $C^{13}$  concentration of 1.11 per cent, again indicating no exchange.

The deficient preparation plus  $Mg^{++}$  decarboxylates oxalacetate completely to pyruvate and carbon dioxide; *i.e.*, no oxalacetate remains as determined by the aniline-citrate method. However, when fumarate is oxidized by this same preparation, some oxalacetate remains, as determined by the aniline-citrate method. Apparently the oxalacetate that is produced by fumarate oxidation differs from that synthesized in the laboratory. The decarboxylation of the physiological oxalacetate may be reversible to such an extent that a detectable quantity of oxalacetate remains, whereas with oxalacetate, as synthesized in the laboratory, no such equilib-

TABLE II

*Exchange of Heavy Carbon Dioxide in Carboxyl Groups*

The  $C^{13}$  concentration of the original bicarbonate in Column 1 was 7 per cent; in all others, 9 per cent. All readings are measured in per cent  $C^{13}$ .

Oxalacetate (from fumarate), carboxyl group adjacent to methylene group, 60 min.	Pyruvate carboxyl, 90 min.	Lactate carboxyl, 210 min.	Pyruvate carboxyl, from lactate oxidation, 210 min.	$\alpha$ -Ketoglutarate, carboxyl group adjacent to carbonyl group, 210 min.
(1)	(2)	(3)	(4)	(5)
1.47*	1.12	1.12	1.12	1.11

\*  $C^{13}$  value when exchange has taken place.

rium occurs. In order to study the exchange reaction with this physiological oxalacetate, oxidation of fumarate was carried out in the presence of  $NaHC^{13}O_3$  (Table II, Column 1). The oxalacetate produced from fumarate was decarboxylated with aniline-citrate. The  $C^{13}$  content of this carboxyl group was 1.47 per cent, or 34.8 per cent above the normal complement. The original  $NaHC^{13}O_3$  in this experiment was prepared from 7 per cent  $C^{13}O_2$ , while in all other experiments reported 9 per cent  $C^{13}O_2$  was used. The amount of exchange was greater than in those experiments in which synthesized oxalacetate was employed. This indicates that exchange is greatly facilitated by the physiological oxalacetate.

The acetone preparation oxidizes pyruvate to acetate and carbon dioxide. A determination as to whether an exchange occurs during this reaction was made under conditions similar to those of the other experiments. The concentration of  $C^{13}$  in the carboxyl group of the pyruvate was 1.12 per cent (Table II, Column 2). Apparently there was little or no exchange of  $CO_2$ .

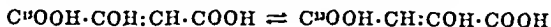
As was stated previously, pyruvate as such may not be the physiologically active compound concerned in the fixation reaction, and since oxalacetate enzymatically derived from the oxidation of fumarate gives a higher exchange value than synthesized oxalacetate, it was considered possible that pyruvate derived from lactate oxidation would bring about an appreciable exchange. The acetone preparation oxidizes lactate to pyruvate, acetate, and carbon dioxide. The accumulation of pyruvate suggests either that its oxidation is slower than that of lactate or that biological pyruvate is formed with which the decarboxylation is reversible. In the latter case the pyruvate would never be completely oxidized. The oxidation of lactate was carried out in the presence of  $\text{NaHC}^{13}\text{O}_3$  until pyruvate accumulated in determinable quantities (210 minutes). The  $\text{C}^{13}$  concentration of the carboxyl group of the pyruvate was 1.12 per cent (Table II, Column 4) and that of the carboxyl group of the residual lactate was 1.12 per cent. These results show that no substantial exchange occurred. The decarboxylation of lactate or pyruvate derived from the lactate is irreversible or at least largely so.

There was no fixation of  $\text{C}^{13}$  in the carboxyl group adjacent to the carbonyl group of  $\alpha$ -ketoglutarate when it was oxidized by the enzyme (Table II, Column 5).

#### DISCUSSION

These experiments were designed to determine whether or not enzymatic and non-enzymatic exchange of  $\text{C}^{13}\text{O}_2$  occurs with the carboxyl groups of keto acids.

During the spontaneous decarboxylation of oxalacetate no appreciable exchange occurs. This indicates that the spontaneous reaction is irreversible. If the small  $\text{C}^{13}$  concentrations indicate reversibility, the rate of carboxylation is extremely slow. In the presence of the enzyme the rate of exchange in oxalacetate is fairly rapid. That there is no exchange, or very little, in the carboxyl group adjacent to the carbonyl group, is extremely interesting in view of the results of Slade *et al.* (15, 16) and Wood *et al.* (3). The former, working with several species of heterotrophic bacteria, and the latter with pigeon liver observed the presence of fixed carbon dioxide in the carboxyl group of lactic acid. Assuming that the fixation occurred by Reaction 1, these authors gave two alternatives to account for the fixed carbon in lactic acid: (a) the reduction of oxalacetate to a symmetrical molecule, from which the lactate was eventually obtained; (b) a suggestion made by Meyerhof (17) wherein the non-enzymatic shifting of OH and H in enol oxalacetate would cause the carboxyl groups to lose their orientation with respect to the original carbonyl and methylene groups. The reaction may be represented as follows:

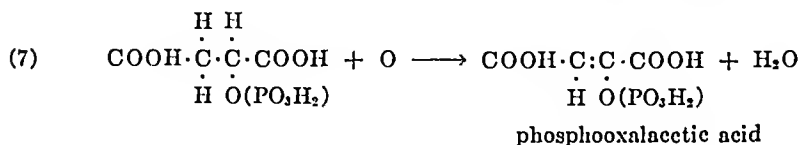
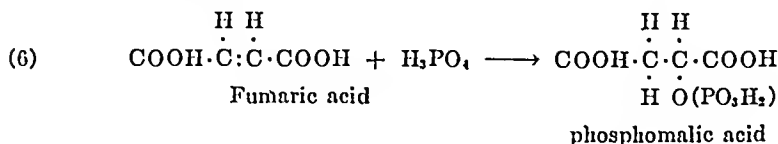


Accordingly the oxalacetate would have fixed  $C^{13}O_2$  in both carboxyl groups, and the occurrence of  $C^{13}$  in the lactic acid would be easily explained. The present experiments do not lend support to this mechanism, since exchange was found to occur only in the carboxyl group adjacent to the methylene group. There is the possibility that the shift of the enolic hydroxyl group may be catalyzed by an enzyme not present in the acetone-treated cells.

Since significant exchange can occur only enzymatically, it is evident that exchange will occur only in those molecules of oxalacetate which are in contact with the enzyme, and are decarboxylated, and then carboxylated. All other molecules of oxalacetate will dilute those in which exchange has taken place, since their  $C^{13}$  concentration will be normal. This explanation would account for the low  $C^{13}$  concentration in the carboxyl group as compared to the bicarbonate. The actual degree of exchange in the molecules making contact with the enzyme probably would be much greater than the results indicate.

When fumarate was oxidized in the presence of  $NaHC^{13}O_3$  (Table II, Column 1), the resulting oxalacetate had a greater  $C^{13}$  value than when exchange was measured with synthesized oxalacetate (Table I). Two possibilities may be suggested to explain this greater exchange: (1) A greater proportion of the oxalacetate molecules obtained from fumarate oxidation was in intimate association with the carboxylating enzyme. This would imply that the fumarate (malate) dehydrogenase is in close proximity to the carboxylating enzyme, thereby insuring contact with the latter enzyme. (2) The oxalacetate obtained from fumarate oxidation is of a different form than that synthesized in the laboratory. The latter explanation appears to be the more likely, since the synthesized oxalacetate is completely decarboxylated to pyruvate and carbon dioxide, whereas an equilibrium apparently is established with that obtained from fumarate oxidation.

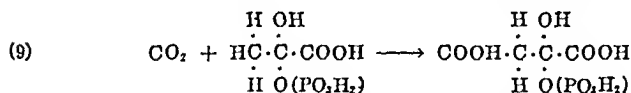
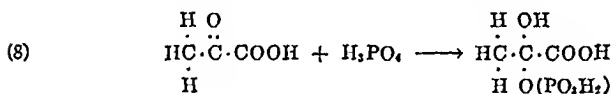
Krampitz, Utter, and Werkman (18) have found that phosphate accelerates the oxidation of fumarate by *Micrococcus lysodeikticus*. It is possible that the oxidation occurs in the manner proposed by Lipmann (19). The reactions are



The phosphooxalacetate may be the physiological compound with which exchange occurs more abundantly. One would expect the phosphooxalacetate to have properties similar to phosphopyruvate but no phosphate fractions with such properties have been obtained from this oxidation.

Solomon *et al.* (20) have shown that  $C^{14}O_2$  is fixed during glycogen synthesis from lactate in rat liver. These authors have proposed that this synthesis occurs by the reversible reactions of the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation. Since the phosphopyruvate + adenylic acid  $\rightarrow$  pyruvate + adenosine triphosphate reaction in this scheme has not been found reversible, they have proposed the synthesis of oxalacetic acid via the fixation reaction (4). The oxalacetate is reduced to fumarate which undergoes Reactions 6 and 7. The phosphooxalacetate is decarboxylated to phosphopyruvate and carbon dioxide. The attainment of the symmetrical fumarate molecule accounts for the fixed carbon in the glycogen.

There is evidence that the utilization reaction requires phosphate; however, the phosphorylation mechanism may be similar to that proposed by Negelein and Brömel (21) for 1,3-diphosphoglyceraldehyde. In this case the phosphoric acid would add to the carbonyl group of pyruvic acid and the  $CO_2$  would react with the resulting compound as follows:



The latter compound becomes phosphooxalacetate (enolic) upon removal of  $H_2O$  from the  $\alpha$ - and  $\beta$ -carbon atoms. The necessity of phosphate in the fixation reaction could thus be explained and phosphopyruvic acid would not be an essential constituent of the reaction. If on the other hand phosphopyruvic acid is found to be essential in the fixation reaction, the mechanism of Solomon *et al.* for glycogen synthesis will have to be revised, for under such conditions the fixation reaction could not be the source of phosphopyruvate for glycogen synthesis. Work is now in progress to determine the phosphate requirements for the exchange reaction, and also whether phosphopyruvate will bring about the carboxylation reaction.

The negative exchange result obtained during the oxidative decarboxylation of pyruvate is not altogether unexpected, since there was no exchange in the carboxyl group adjacent to the carbonyl group in oxalacetate. Ferdman and Epstein (22) have reported the necessity of phosphate for



lactate oxidation; consequently the pyruvate resulting from this oxidation may be phosphorylated and thus be capable of being carboxylated. No oxalacetate could be detected, however, after lactate oxidation and in addition the pyruvate did not exhibit an ability to exchange  $\text{CO}_2$ , furthering the evidence against a  $\text{C}_2$  and  $\text{C}_1$  addition hypothesis.

Wood *et al.* (3) have found  $\text{C}^{13}\text{O}_2$  fixed in the carboxyl group adjacent to the carbonyl group of  $\alpha$ -ketoglutaric acid formed during the dissimilation of pyruvic acid with pigeon liver. They have proposed that the carbon is initially fixed by 3- and 1-carbon addition and that the  $\alpha$ -ketoglutarate is derived from the oxalacetate by the Krebs cycle. That the fixed carbon probably did not arise by the carboxylation of succinic acid is borne out by the negative exchange values observed in the present investigation of the oxidation of  $\alpha$ -ketoglutarate to succinate and carbon dioxide.

#### SUMMARY

The exchange of  $\text{C}^{13}\text{O}_2$  with the carboxyl groups of oxalacetic acid during spontaneous decarboxylation was found to be insignificant. The exchange, however, significantly increased during the enzymatic decarboxylation of the acid. The exchange occurred exclusively in the carboxyl group adjacent to the methylene group.

Evidence is presented to show that a dynamic equilibrium involving a shift of the hydroxyl of enol oxalacetate does not occur. Oxalacetate derived from fumarate oxidation gave high exchange values. No exchange occurred during the oxidative decarboxylation of pyruvate or  $\alpha$ -ketoglutarate, nor did pyruvate derived from lactate oxidation give exchange. These results are further evidence of the validity of the fixation reaction as proposed by Wood and Werkman.

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## LETTERS TO THE EDITORS

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### ACIDOSIS AND DECREASED URINE FLOW IN THE RABBIT DURING GRAVITY SHOCK

*Sirs:*

When rabbits are held vertically with their heads up, they become unconscious, or nearly so, just before respiratory failure, a condition which has been called gravity shock. If returned to the horizontal position at that time, 75 per cent of them recover completely. The other 25 per cent die within 12 hours, even though their posture, respiratory rate, and general behavior were normal during most of that time.

Studies on the heart blood of thirty rabbits before and after gravity shock have revealed in the shocked animals a severe acidosis (pH 6.85 to 7.10), acapnia (50 to 70 per cent decrease in  $\text{CO}_2$ ), marked increases in lactic and pyruvic acids (100 and 20 per cent), and an unusual phosphatemia (150 per cent increase above normal). Simultaneously the urine flow steadily declined to zero. After return to the horizontal position urine flow, in the animals which recovered, gradually increased to the normal rate within a few hours, and the urine showed a significant increase in phosphate content. The pH of the blood also returned to normal in the same time.

These facts are interpreted to mean that hanging of the rabbit reduces blood flow through the peripheral tissues of the posterior regions, causing an oligemic anoxia of those tissues. Lactic, pyruvic, and phosphoric acids, therefore, appear in the blood and increase its acidity, while the carbon dioxide content decreases. When the pH has decreased sufficiently, the formation of urine is stopped, so that the excretion of all substances which are sustaining factors in shock is prevented. Unless the animal can resume urine formation after return to the horizontal position and thus rid the blood of the harmful substances, it will die.

The study of this condition is being extended and a full report of the findings will be made later.

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# XANTHOPTERIN IN THE TREATMENT OF LEUCOPENIA AND WEIGHT LOSS IN RATS FED SUCCINYLSULFATHIAZOLE

Sirs:

Preliminary unpublished data<sup>1</sup> have indicated that xanthopterin (the fish anemia factor)<sup>2</sup> is capable of alleviating the blood changes in nutritional cytopenia in the monkey, but the evidence is not yet conclusive that xanthopterin is identical with "vitamin M." The similarity of the syndromes in the monkey<sup>3</sup> and in succinylsulfathiazole-treated rats<sup>4</sup> led us to believe that xanthopterin might overcome the deficiency in the latter species also. The changes produced in the rat were prevented or cured by treatment with whole liver or liver fractions,<sup>4</sup> some of which have also been found to be active in the prevention of vitamin M deficiency in the monkey.<sup>3</sup> Likewise "folic acid" has been found to be at least partially effective in overcoming the deficiency produced by feeding succinylsulfathiazole.<sup>5</sup> In a single experiment by Wilson *et al.* it was reported that a folic acid concentrate raised the leucocyte count of a vitamin M-deficient monkey.<sup>6</sup>

In this note we wish to report the effectiveness of xanthopterin in alleviating the leucopenia and counteracting the growth inhibition produced by succinylsulfathiazole.

Rats 25 days old from our stock colony were used. The basal ration had the following percentage composition: sucrose 74, casein 18, cottonseed oil 3, salts<sup>7</sup> 2, cod liver oil 2, and succinylsulfathiazole<sup>8</sup> 1. The B vitamins were fed daily in supplement dishes at the following levels: thiamine chloride 50  $\gamma$ , riboflavin 34  $\gamma$ , pyridoxine 20  $\gamma$ , calcium pantothenate 100  $\gamma$ , and nicotinamide 200  $\gamma$ . After 5 weeks, at which time growth had ceased, 0.05 cc. of a biotin concentrate (S. M. A., No. 1000) was included in the vitamin supplements of all animals. 20  $\gamma$  of xanthopterin daily were given at this time to six of the eleven experimental ani-

<sup>1</sup> Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., unpublished experiments.

<sup>2</sup> Simmons, R. W., and Norris, E. R., *J. Biol. Chem.*, **140**, 679 (1941).

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<sup>5</sup> Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 713 (1942).

<sup>6</sup> Wilson, H. E., Doan, C. A., Saslaw, S., and Schwab, J. L., *Proc. Soc. Exp. Biol. and Med.*, **50**, 341 (1942).

<sup>7</sup> Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, **14**, 273 (1937).

<sup>8</sup> Succinylsulfathiazole was kindly supplied by Sharpe and Dohme, Inc., Glendolen, Pennsylvania.

mals. The xanthopterin used was synthesized by a modification of the Purrmann method<sup>9</sup> developed at this laboratory.

The five control animals receiving biotin showed a slight growth response followed by a continued loss of weight, while those receiving both biotin and xanthopterin showed an immediate weight gain and a pronounced leucocyte response. The average white cell count after 5 days of xanthopterin therapy was 9400 white cells per c.mm., while in the control group the average was only 3420 per c.mm.

If the deficiency produced by succinylsulfathiazole is of a specific nature, then it seems probable that the "folie acid" concentrates used by Nielsen and Elvehjem<sup>5</sup> contained xanthopterin or some closely allied substance. The possibility that folie acid is identical with xanthopterin should not be overlooked. On the other hand, this substance may not be the only one involved in protection against succinylsulfathiazole. Preliminary studies indicate that the distribution of white blood cells is not normal after xanthopterin therapy and that normal growth is not fully restored; whether these results should be interpreted as showing the lack of other essential substances or whether they indicate an injurious action of the drug is not yet clear.

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<sup>9</sup> Purrmann, R., *Ann. Chem.*, 546, 98 (1940).

## THE EXCRETION OF PREGNANEDIOL FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE TO RABBITS\*

Sirs:

Cuyler *et al.*<sup>1</sup> reported that a healthy man excreted 29 and 16 mg. of sodium pregnanediol glucuronide after receiving intramuscularly a total of 25 and 50 mg., respectively, of desoxycorticosterone acetate. These authors were unable to confirm their original observation in three other men,<sup>2</sup> or to demonstrate any clear cut augmentation in the excretion of sodium pregnanediol glucuronide in a group of women receiving desoxycorticosterone acetate.<sup>3</sup> Because of these conflicting observations we were led to reinvestigate the possible conversion of desoxycorticosterone to pregnanediol.

The rabbit was chosen as the test animal, since this species, which normally does not excrete detectable amounts of pregnanediol, excretes pregnanediol glucuronide after the administration of progesterone.<sup>4</sup> Crystalline desoxycorticosterone acetate dissolved in oil was injected subcutaneously in amounts varying from 225 to 930 mg. over a period of 1 to 10 days to each of eight adult rabbits. The urine collected during the injection period and for the succeeding 3 days was extracted daily with butyl alcohol. The combined butanol extracts were examined by Venning's method.<sup>5</sup> The results are shown in the table.

In each instance a precipitate, indistinguishable from sodium pregnanediol glucuronide as to method of isolation and melting point, was obtained. Conclusive identification was established by the isolation of pregnanediol from the acid hydrolysates of the precipitates isolated in Experiments 2 and 6. Hydrolysis of 100 mg. of the precipitate from Experiment 2 yielded 38 mg. of a product melting at 220–225° which, after chromatographic analysis and two crystallizations, melted at 235–236° and at

\* The authors are indebted to Dr. E. Sehwenk of the Schering Corporation for the desoxycorticosterone used in these experiments and to Mrs. D. Jewitt of Ayerst, McKenna and Harrison, Ltd., for the microanalyses.

<sup>1</sup> Cuyler, W. K., Ashley, C., and Hamblen, E. C., *Endocrinology*, **27**, 177 (1940).

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236-237° on admixture with authentic pregnane-3( $\alpha$ ),20( $\alpha$ )-diol. Found, C 78.77, H 11.42; calculated for  $C_{21}H_{36}O_2$ , C 78.75, H 11.25. Thus 5.6 to 14.6 per cent of the desoxycorticosterone injected was excreted as pregnanediol. The extent of this interconversion is of the same order as that previously demonstrated for the conversion of progesterone to pregnanediol in the rabbit.<sup>4</sup>

Experiment No.	Test animal	Desoxycorticosterone acetate administered		Pregnanediol glucuronide isolated		Desoxycorticosterone converted to pregnanediol
		Period of injection	Amount	Weight	M.p.	
		days	mg.	mg.	°C.	per cent
1	M.	3	482	35.2	254-257	5.7
2	"	3	930	140	259-263	10.5
3	"	3	225	18.2	262-267	5.6
4	"	1	270	48.4	254-260	12.5
5	"	10	345	38.4	264-268	7.7
6	F. Isolated	3	460	65.3	256-261	9.8
7	" Pregnant	3	500	63.3	264-267	8.8
8	" "	3	520	109.1	258-262	14.6

Under certain circumstances part of the pregnanediol in human urine may originate from the adrenal cortex.<sup>6</sup> In view of the above results it seems likely that pregnanediol of adrenal origin might arise not only from progesterone,<sup>7</sup> but also from desoxycorticosterone, which has also been isolated from the adrenal cortex.<sup>8</sup>

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<sup>9</sup> Supported by a grant from the Banting Research Foundation.

## THE STATE OF PANTOTHENIC ACID IN BLOOD

Sirs:

Stanbery, Snell, and Spies,<sup>1</sup> by employing slight modifications in technique, have successfully applied the microbiological assay method for pantothenic acid of Pennington, Snell, and Williams<sup>2</sup> to the determination of pantothenic acid in blood. They reported that normal human blood contains between 0.19 and 0.32  $\gamma$  of pantothenic acid per cc. (average, 0.225  $\gamma$  per cc.). These results have been confirmed by others<sup>3</sup> when *Lactobacillus casei*  $\epsilon$  was used as the test organism. However, Pelczar and Porter<sup>4</sup> in assaying *clarified* blood for pantothenic acid, by a method depending on the essential nature of pantothenic acid for *Proteus morganii*, found human blood to contain only 0.032 to 0.099  $\gamma$  of pantothenic acid per cc. An explanation for this apparent discrepancy is believed to be found in the results of the following experiments shown in the table.

In keeping with the experience of others, we found whole blood or plasma of various species (human, rabbit, and dog) to contain 0.15 to 0.35  $\gamma$  of pantothenic acid per cc. when assayed by the method of Pennington *et al.*<sup>2</sup> Protein-free filtrates of whole blood prepared with tungstic acid (Folin-Wu procedure) or zinc hydroxide (Somogyi) were found to contain an amount of pantothenic acid equivalent to 0.030 to 0.070  $\gamma$  per cc. of original blood. Pantothenic acid, when added to whole blood and determined with or without protein precipitation, or when added to blood filtrates, could be quantitatively recovered. The low assay results with blood filtrates are therefore not due to adsorption of pantothenic acid. When unheated whole blood or plasma was added aseptically to tubes of sterile medium and then inoculated with *Lactobacillus casei*, acid production indicated an amount of pantothenic acid corresponding to 0.030 to 0.050  $\gamma$  per cc. Assay of the blood or plasma after heat sterilization in the usual manner gave values of 0.15 to 0.30  $\gamma$  of pantothenic acid per cc.

These results are believed to demonstrate the existence in blood of pantothenic acid in at least two states. The major portion exists in a "combined" state and is precipitable by protein precipitants. When blood or plasma is assayed without heat treatment *Lactobacillus casei*  $\epsilon$

<sup>1</sup> Stanbery, S. R., Snell, E. E., and Spies, T. D., *J. Biol. Chem.*, **135**, 353 (1940).

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<sup>3</sup> Pearson, P. B., *J. Biol. Chem.*, **140**, 423 (1941). Strong, F. M., Feeney, R. E., and Earle, A., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 566 (1941).

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Sample No.	Material assayed	Heat treatment	Pantothenic acid found	Recovery of added pantothenic acid
			$\gamma$ per cc.	per cent
1	Whole blood	Autoclaved	0.20	
1	Plasma	"	0.21	
1	Folin-Wu filtrate		0.023*	
1	Somogyi filtrate		0.030*	
2	Whole blood	Autoclaved	0.41	
2	Folin-Wu filtrate		0.070*	
2	" " + added pantothenic acid (0.01 $\gamma$ per cc. filtrate)			110
2	Whole blood + added pantothenic acid (0.1 $\gamma$ per cc. blood) followed by Folin-Wu pptn.			101
3	Whole blood	Autoclaved	0.23	
3	Folin-Wu filtrate		0.038*	
3	Whole blood + added pantothenic acid (0.1 $\gamma$ per cc. blood) followed by Folin-Wu pptn. at once			107
3	Same, but pptd. after 48 hrs. at room temperature			97
4	Whole blood	Autoclaved	0.15	
4	" "	Unheated	0.073	
4	Folin-Wu filtrate		0.066*	
5	Plasma	Autoclaved	0.32	
5	"	Unheated	0.034	

\* Calculated on the basis of the original blood.

responds only to the "free" pantothenic acid present. Heat sterilization renders the "combined" pantothenic acid available to this organism.

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## PHOSPHORYLATED CARBOHYDRATE ESTERS IN AUTOTROPHIC BACTERIA

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Strictly autotrophic bacteria are microorganisms which grow only in the presence of a specific inorganic nutrient from the oxidation of which they obtain their energy and which are able to cover all of their carbon needs from  $\text{CO}_2$ . In most cases organic materials are not utilized and may be toxic. One representative of this group of organisms, *Thiobacillus thiooxidans*, oxidizes sulfur to sulfuric acid as its energy source and no material yet found can replace  $\text{CO}_2$  as a source of carbon for growth. This organism is further remarkable in that it will only grow at a pH of below 6, and produces, during its growth, as much as 5 per cent sulfuric acid without detectable harm to its metabolism. It has recently been shown that cells of this organism were able to survive relatively long periods in the absence of oxidizable sulfur and that under these circumstances it possessed an organic metabolism resulting in the liberation of  $\text{CO}_2$  (1). LePage (2) was able to demonstrate that during this "endogenous metabolism" a polysaccharide storage product was broken down and O'Kane (3), Vogler and Umbreit (4), and LePage (2) have provided evidence that phosphorylation was somehow involved in this breakdown. It was therefore of considerable interest to determine whether this metabolism followed a pathway similar to that of yeast and muscle or whether a different, and perhaps more primitive, type of phosphorylating metabolism was involved.

Two limitations are imposed upon studies of this organism. First, the difficulty of obtaining sufficient amounts of tissue for experiment necessitates the application of micromethods throughout. Second, the usual methods of study of bacterial metabolism are not applicable, since organic materials are not utilized by this organism; *i.e.*, one cannot supply an external source of glucose, for example, and determine the fermentation products.

It was therefore necessary to modify existing methods for the determination of the phosphorylated carbohydrate esters and to apply them on a micro scale. In the procedure employed most of the compounds are determined by at least two independent measurements. By application of this method it was possible to account for over 90 per cent of the acid-extractable phosphorus of the autotrophic cell in terms of known compounds whose identity is clearly established. The results showed that the

following compounds were present in the extract of *Thiobacillus thiooxidans*: adenosine triphosphate, fructose-1,6-diphosphate, phosphoglyceric acid, fructose-6-phosphate, glucose-6-phosphate, and glucose-1-phosphate. Presumptive evidence is offered for the presence of coenzyme I.

If it is granted that the presence of these compounds (which have been synthesized from  $\text{CO}_2$ ) indicates that they function in the metabolism of the organism, then the autotrophic bacteria, in spite of their unusual conditions of growth, have an internal metabolism similar to, if not identical with, the phosphorylating glycolyses found in yeast and muscle. This paper also contains evidence that these compounds do function in the metabolism, in that the relative amount of each material varies with the physiological condition of the cells. For purposes of brevity only one analysis is described but changes in the distributions under physiological conditions are noted when they occur.

#### EXPERIMENTAL

The methods consist essentially of an intermittent trichloroacetic acid extraction of the wet bacterial cells until no more phosphorus is removed and a separation of the combined extracts into three rather well defined fractions. Each fraction is then analyzed for individual compounds known to occur in it; at least two characteristic properties of the compound to be determined are employed in an attempt to account for all of the phosphorus in the fraction. A flow diagram of the method of obtaining the fractions is presented, together with the data obtained in the experiment described. This fractionation method is a combination and modification of those of Eggleton and Eggleton (5), Needham and van Heyningen (6), O'Kane (3), and O'Kane and Umbreit (7).

When applied to other organisms, the alkali treatment (which destroys a large part of the triose phosphate and phosphopyruvic acid) is usually not necessary, but in *Thiobacillus thiooxidans* it is not possible to extract any appreciable amount of phosphorus if this treatment is omitted. Analytical methods for various components of the fractions were modified and adapted to the Evelyn photoelectric colorimeter from the following methods: *total and inorganic phosphorus* based on that of Fiske and Subbarow (8) and described in more detail by LePage (2), *nitrogen* after the method of Johnson (9), *fructose* after the method of Roe (10), *pentose* after the method of Mejbaum (11), *phosphoglyceric acid* after the method of Rapoport (12), *phytic acid* after the method of Rapoport *et al.* (13), *carbazole reactions* after the method of Gurin and Hood (14), and *reducing values* after the method of Folin and Malmros (15). These methods were standardized with pure compounds when necessary, as described in the text. *Hydrolysis curves* were run in 1 N HCl at  $100^\circ$ , according to the

method of Lohmann (16). Cells from a 7 day culture were employed and were grown on an inorganic medium with sulfur as the sole energy source and harvested as described by Vogler (1).

*Method of Obtaining Organic Phosphorus Fractions*

2.35 gm. (dry weight) bacterial cells (0.235 gm. N, 47.5 mg. total P) in 15 ml., treated with 1.5 ml. 50% KOH for 5 minutes, neutralized with 100% trichloroacetic acid; 2.5 ml. 100% trichloroacetic acid added to 25 ml.; extracted 12 hours, 0-5°; centrifuge

Cell debris extracted with 20 ml. 10% tri-chloroacetic acid, 12 hours, 0-5°; centrifuge

Cell debris; repeat extraction

Cell debris; repeat extraction

Cell debris; repeat extraction

Cell debris contains 8.8 mg. total P (18.7% of original); considered acid-insoluble

Extract contains no P; discarded

Extracts combined; made to 100 ml.; contain 5.86 mg. inorganic P (12.3% of original); 32.84 mg. organic P (69.0% of original)

Aliquot containing 28.05 mg. organic P and 5.0 mg. inorganic P; treated with 2.7 ml. 25% barium acetate and adjusted to pH 8.4 with NaOH. Let stand at 0°, 30 minutes; centrifuge

Add slight excess  $H_2SO_4$ ; centrifuge

$BaSO_4$ ; wash; centrifuge

$BaSO_4$ ; discard

Add barium acetate and NaOH, to pH 8.2; 0°, 30 minutes; centrifuge

*Barium-insoluble fraction (I)*  
(Redissolved and reprecipitated without removing any further P.) Ba removed with  $H_2SO_4$ , made to 75 ml. Contained 6.5 mg. organic P (23.7% of original organic P); 3.38 mg. inorganic P

Combined extracts treated with 5 volumes 95% ethanol; 3 hours, 0-5°; centrifuge

$H_2SO_4$  to remove Ba;  $BaSO_4$  washed and original and washings made to 150 ml.

Concentrated to small volume under vacuum, 30-35°; Ba removed

*Barium-soluble alcohol-insoluble fraction (II)*  
19.0 mg. organic P (67.6% of original organic P); 0.545 mg. inorganic P

*Barium-soluble alcohol-soluble fraction (III)*  
0.965 mg. organic P (3.43% of original organic P); 0.0 mg. inorganic P

Recoveries: organic P 94.7%; inorganic P 78.6%; low recoveries of inorganic P due to adsorption on numerous  $BaSO_4$  ppts.

The compounds present in the fractions obtained as shown in the diagram were as follows:

*Barium-Insoluble Fraction (I)*—75 ml., contained 85.4  $\gamma$  of organic P per ml.

*Adenosine Triphosphate (ATP) Hydrolysis*—The hydrolysis curve for the entire fraction is given in Fig. 1, Curve A. When the phosphorus released in 7 minutes was calculated as ATP (after Lohmann (16)), 88.9 per cent of the organic phosphorus in this fraction was ATP.

*Nitrogen*—57.0  $\gamma$  of nitrogen were found per ml. If this nitrogen were all ATP, 88.8 per cent of the organic phosphorus in this fraction would be ATP. Proof that the only nitrogen compound present was ATP was obtained as follows: Upon 7 minutes hydrolysis in 1  $\times$  HCl at 100° ATP breaks down to adenine, pentose phosphate, and adenylic acid (17), all of which are barium-soluble. The inorganic phosphorus, phosphoglyceric acid, and undecomposed hexose diphosphate can be removed by barium precipitation. The filtrate should then contain all of the nitrogen of the ATP but only 1 phosphorus, *i.e.* the N:P ratio should be 2.26; found 2.25; if corrected for hexose diphosphate breakdown (26.5 per cent in 7 minutes), the ratio found was 2.26. This provides adequate proof that the nitrogen observed was present as ATP. It is of some interest that the hydrolysis

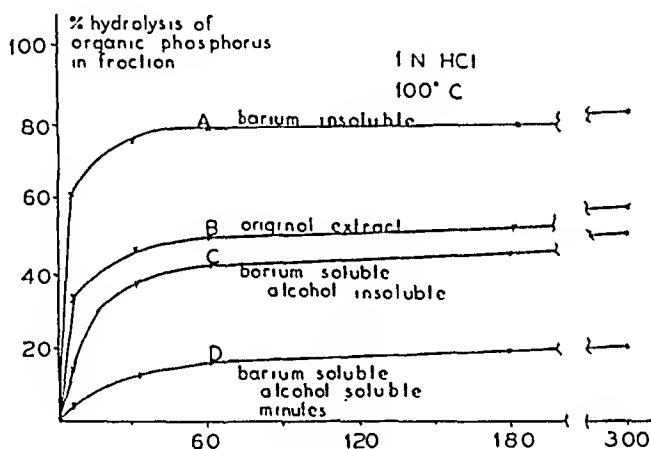


FIG. 1. Hydrolysis curves

products of ATP are themselves fairly resistant to hydrolysis (in 1  $\times$  HCl at 100° for 1 hour 28.7 per cent was hydrolyzed; in 2 hours, 50.7; in 3 hours, 58.7 per cent).

*Pentose*—119.7  $\gamma$  of pentose were found per ml. If this pentose resided in the molecule of ATP, 87 per cent of the organic phosphorus of this fraction would be ATP.

*Hexose Diphosphate (Fructose-1,6-diphosphate) Hydrolysis*—The hexose diphosphate content of this fraction is too low to permit accurate calculation of its content from the hydrolysis curves.

*Fructose*—5.32  $\gamma$  of fructose were found per ml. If this fructose resulted from the hexose diphosphate, 1.13 per cent of the organic phosphorus of this fraction is hexose diphosphate.

*Reducing Value*—1.13 per cent of the organic phosphorus of this fraction

was calculated from the reducing value of 0.954  $\gamma$  of glucose activity per ml., as follows: With the Folin and Malmros method (15) of determining reducing values the labile phosphorus esters are not hydrolyzed, since the pH is alkaline and the heating time is short. Tests showed no reaction with pure ATP or with glucose-1-phosphate. The data on the reduction values of hexose diphosphate in the literature have been obtained by more drastic methods; it was therefore necessary to determine the reducing value of pure hexose diphosphate by this method. Purified hexose diphosphate was obtained from the Schwartz Laboratories, Inc., New York, as the barium salt. After the removal of the barium, the ester should contain 18.22 per cent organic phosphorus; found 18.26 per cent. The hydrolysis curve in 1  $N$  HCl at 100° is identical with that given in the literature (18). This pure hexose diphosphate gave a reduction value, with the Folin and Malmros method, 9.5 per cent that of glucose.

*Phosphoglyceric Acid Hydrolysis*—The resistant phosphorus (3 hours hydrolysis) represents 19.1 per cent of the total organic phosphorus. In addition to the phosphoglyceric acids, however, it also contains the pentose phosphates from ATP. Data on their resistance (see ATP, above) indicate that they are 58.7 per cent hydrolyzed in 3 hours. This would account for 12.2 per cent of the organic phosphorus of this fraction, leaving 7.2 per cent presumably due to the phosphoglyceric acids.

*Rapoport Method*—Fortunately there is a rather specific and accurate method for the determination of phosphoglyceric acids (12). This method was modified, adapted to the photoelectric colorimeter, and standardized by the use of a sample of pure phosphoglyceric acid obtained from Professor W. H. Peterson. Measurement of optical rotation indicated that 95 per cent of this material was 3-phosphoglyceric acid. There were 46.4  $\gamma$  of phosphoglyceric acid per ml. of the fraction determined by this method. This represents 9.05 per cent of the organic phosphorus of the fraction.

*Physiological Variation*—The organic phosphorus of this fraction (88.8 per cent ATP, 1.1 per cent hexose diphosphate, 9.0 per cent phosphoglyceric acids) is thus satisfactorily accounted for by the three components studied (99.0 per cent). The fraction was also examined for phytic acid by the method of Rapoport *et al.* (13) but none was found. These cells have a relatively high ATP content and were very active in the oxidation of sulfur ( $QO_2$  (N) 3590); *i.e.*, they were physiologically "young" cells (*cf.* Vogler (19)). If these cells are allowed to respire until the  $QO_2$  (N) begins to drop (2), there is a decrease in the amount of ATP and an increase in the amount of hexose diphosphate.

*Barium-Soluble, Alcohol-Insoluble Fraction (II)*—150 ml., contained 129.2  $\gamma$  of organic P per ml.

*Glucose-1-phosphate (Cori Ester) Hydrolysis*—The hydrolysis curve for



the entire fraction is given in Fig. 1, Curve C, in which it is apparent that acid-sensitive phosphorus is present. This could arise from glucose-1-phosphate, phosphopyruvic acid, or unknown constituents. Phosphopyruvic acid is probably not present because of the alkali treatment given the cells. If the hydrolysis at 7 minutes is calculated as glucose-1-phosphate, 15.65 per cent of the organic phosphorus is due to this material. This value is undoubtedly high, inasmuch as the other esters in this fraction, while more resistant to acid hydrolysis, are much in predominance, and hence contribute to the easily hydrolyzable phosphorus.

*Increase in Reducing Value*—The reducing value of pure synthetic glucose-1-phosphate obtained as the potassium salt from Dr. S. P. Colowick (P content, calculated 9.23 per cent; found 9.03) by the Folin and Malmros method was 0 for the unhydrolyzed material and 66.4 per cent that of glucose after 7 minutes hydrolysis (calculated upon the basis of the salt of 98 per cent purity). Since glucose-6-phosphate and fructose-6-phosphate are already reducing without hydrolysis, it would seem that a measure of the 1-ester could be obtained by measurement of the increase in reducing value on 7 minutes hydrolysis (20). The other esters contribute less than 1 per cent of the increased reducing value. If the increase in reducing value after 7 minutes hydrolysis is calculated as glucose-1-phosphate, 9.92 per cent of the organic phosphorus would be glucose-1-phosphate.

*Fructose-6-phosphate (Neuberg Ester)*—The total reducing value before hydrolysis was equivalent to 95.4  $\gamma$  of glucose per ml. Fructose, determined by the method of Roe (10), was equivalent to 107.8  $\gamma$  of fructose per ml. This method was standardized by the use of pure fructose-6-phosphate (free acid, P calculated 11.9 per cent; found 11.92) obtained from hexose diphosphate by acid hydrolysis, barium separation, and repeated alcohol precipitation of the barium salt. Pure fructose-6-phosphate has a reducing value of 31.65 per cent that of glucose when not hydrolyzed; hence the fructose found in this fraction is equivalent to 34.1  $\gamma$  of glucose per ml., or 9.94 per cent of the organic phosphorus of this fraction.

*Glucose-6-phosphate (Robison Ester)*—If the residual reducing value is due to glucose-6-phosphate, this ester constitutes 42.7 per cent of the organic phosphorus of this fraction. This is based upon the reducing value of pure glucose-6-phosphate (prepared as described below) of 13.2 per cent that of glucose.

To obtain further evidence of the presence of the Neuberg and Robison esters, an aliquot of the fraction was subjected to 7 minutes hydrolysis to destroy the glucose-1-phosphate, barium fractionation, and alcohol precipitation of the barium filtrate. The phosphate from the glucose-1-phosphate was removed in the barium precipitate, the glucose in the alcohol filtrate. The reducing value was found to be 98.5 per cent of the value

before this treatment; i.e., the initial reducing value ascribed to the 6-esters has remained essentially unchanged by this purification.

Since glucose-6-phosphate seemed to constitute a large part of this fraction, it was isolated as follows: An aliquot of the fraction was hydrolyzed in 1 N HCl at 100° for 12 hours. This destroys all of the glucose-1-phosphate and fructose-6-phosphate and most other esters (including pentose phosphates and coenzymes) together with 25 to 30 per cent of the glucose-6-phosphate. The hydrolysate was then fractionated with barium to remove the inorganic phosphorus and the remaining glucosc-6-phosphate precipitated from the filtrate with 5 volumes of 95 per cent alcohol. Several alcohol precipitations were employed to purify this material, which then showed 30.0  $\gamma$  of organic phosphorus per ml., no inorganic phosphorus, and a reducing value equivalent to 35.5  $\gamma$  of glucose per ml. If the phosphorus were all glucosc-6-phosphate, the reducing value would be 13.3 per cent that of glucosc. Pure glucose-6-phosphate obtained in a like manner from a mixture of the monophosphates from yeast (P, calculated as glucose-6-phosphate, 11.9 per cent; found 11.83) showed a reducing value of 13.2 per cent. Thus the material isolated was glucose-6-phosphate. The amount of this ester actually isolated was 27 per cent of that estimated to be present.

*Coenzyme I*—So far only 62.6 per cent of the organic phosphorus of the fraction has been accounted for by the three compounds determined. In view of the presence of mannose in the polysaccharide storage product (2), it is of interest that the carbazole reaction of Gurin and Hood (14) indicated that the only hexoses present were fructose and glucose. In these determinations it was noted that the absorption maximum was shifted, indicating the presence of pentoses. Accordingly, pentose was estimated, showing 253  $\gamma$  of pentose per ml. It is also known that coenzymes I and II and adenylic acid (or its split-products) can occur in this fraction. The nitrogen content was found to be 80  $\gamma$  of nitrogen per ml. The pentose-nitrogen-organic phosphorus relation of this fraction (with phosphorus unaccounted for as the phosphorus value) is 150:47.5:28.7, which points definitely to coenzyme I (pentose-N-P; adenylic acid, 150:70:31; coenzyme I, 150:49:31; coenzyme II, 150:49:46.5). This possibility was not studied further. If the pentose content observed was due to coenzyme I, 40.5 per cent of the organic phosphorus of the fraction was accounted for by this material.

*Physiological Variation*—If cells are allowed to respire without sulfur until the  $QO_2$  (N) on sulfur begins to drop, there is a tendency for the glucose-1-phosphate to increase. In a sample in which the  $QO_2$  (N) had dropped to 2295, 18.6 per cent of the alcohol-precipitable fraction was glucose-1-phosphate. It should also be noted that there is a greater quan-

tity of monophosphates in these phosphorus distributions than is generally found in yeast or muscle and that the quantities of the various esters are not in strict equilibrium proportions.

*Barium-Soluble, Alcohol-Soluble Fraction (III)*—This fraction, constituting 3.43 per cent of the acid-extractable organic phosphorus of the cell, could not be further fractionated because of the relatively small amount of phosphorus and the presence of accumulated salts. The hydrolysis curve (Fig. 1, Curve D) shows that most of the material was relatively resistant to acid hydrolysis.

TABLE I

*Chemical Composition of Phosphorus Compounds of Thiobacillus thiooxidans*

Fraction	P found, per cent of total P in cells		
Acid-insoluble .....	18.7		
Inorganic .....	12.3		
Acid-soluble organic .. .. .	69.0		
Fractions of acid-soluble organic P			
	P found, per cent of fraction	P found, per cent of total acid-soluble organic	
I. Barium-insoluble.....		23.7	
Adenosine triphosphate....	88.8		21.1
Hexose diphosphate.....	1.1		0.2
Phosphoglyceric acids .....	9.0		2.1
	99.0		
II. Barium-soluble, alcohol-insoluble		67.6	
Glucose-1-phosphate.....	9.9		8.7
Glucose-6-phosphate.....	42.7		28.9
Fructose-6-phosphate.....	9.9		8.7
Coenzyme I.....	40.5		27.0
	103.0		
III. Barium-soluble, alcohol-soluble (not further fractionated) ...		3.4	
Recovery .....		94.7	

## SUMMARY

A summary of the fractionation of the phosphorus compounds described in the text for the autotrophic bacterium *Thiobacillus thiooxidans* is given in Table I. From this it is apparent that a large portion of the acid-extractable phosphorus is composed of phosphorylated carbohydrate esters identical in chemical properties with those of yeast and muscle. If it is granted that the presence of these compounds in an autotrophic cell (which has synthesized them from CO<sub>2</sub>, using energy from the oxidation of sulfur) means that they play an important rôle in the metabolism of this

cell, it is possible to conclude that the internal metabolism of the autotrophic cell is markedly similar to, if not identical with, heterotrophic tissues.

This work was supported by the Wisconsin Alumni Research Foundation. The authors are greatly indebted to Lieutenant D. J. O'Kane, whose studies on this problem were terminated by the war, to Dr. S. P. Colowick for the glucose-1-phosphate, to Dr. W. H. Peterson for the phosphoglyceric acid, and to Dr. V. R. Potter for the mixed monophosphate esters from yeast.

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# FRACTIONAL DISTILLATION OF UNSATURATED FATTY ACIDS

## II. THE EFFECT OF HEAT ON THE REARRANGEMENTS PRODUCED IN UNSATURATED FATTY ACID ESTERS\*

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In a previous publication (1) we have reported on the heat sensitivity of unsaturated fatty esters from cod liver oil as determined by chemical and spectroscopic methods. The present communication amplifies and extends this work to include the more common unsaturated fatty acids found in animal and vegetable fats.

Throughout all this work the primary aim has been to obtain quantitative measurements of the amount of change produced in unsaturated esters as a result of the heating necessarily involved in their fractional distillation. This is best done by noting the increase in absorption at certain wavelengths, 2350 and 2700 Å., known to be correlated with the appearance of two and three double bond conjugation respectively, since the development of conjugation is probably the most fundamental change involved.

Measurements of increased light absorption are of little value, however, unless they can be compared to some upper limit of absorption. In the past this has been done by comparing the specific extinction coefficients of ester fractions and residues with those of pure conjugated fatty acids containing an equal number of double bonds. Such a comparison is likely to be misleading, since it has not been shown to date that heat treatment will cause conjugation of all of the double bonds in any given unsaturated ester. Rather, it appears that only a relatively small amount of conjugation can be produced before polymerization reactions remove conjugated material as fast as it is formed. It is necessary, therefore, to determine the maximum amount of conjugation attainable under specified heating conditions. A measured conjugation of 5 per cent in the pot liquid midway through a fractional distillation, for example, may take on a new significance if it is found that only a 10 per cent conjugation can be produced by the most strenuous heating conditions. For this reason, we have studied the effect of time and temperature on the development of

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conjugation in unsaturated esters of cod liver oil; and although, as was expected, complete conjugation could not be obtained, the results furnish additional information concerning the mechanism of polymerization, and lend further credence to Scheiber's conjugation hypothesis (2).

In the experimental part which follows the effect of heat on highly unsaturated esters of fatty acids, such as are prepared from cod liver oil, is taken up first. This is followed by data on the less unsaturated esters of fatty acids prepared from linseed oil and linolenic acid.

#### EXPERIMENTAL

*Preparation of Unsaturated Methyl Esters of Cod Liver Oil*—The esters (iodine number 179) used for the heating experiments at 150° and 250° were prepared from cod liver oil acids largely freed of solid acids by cooling a 10 per cent solution in acetone to -20° and discarding the precipitate. Esters of iodine number 176, similarly prepared, were used for the heating experiment at 200°. Details of these preparations may be found in Paper I (1).

*Heat Treatment of Unsaturated Esters of Cod Liver Oil*—These esters were heated at 150°, 200°, and 250° in the following way: A suitable quantity of the esters was placed in a 2-necked flask equipped with a gas inlet tube attached at the bottom. Nitrogen was continually forced through the solution and out the long condenser tube in the side neck. The central neck contained a thermometer whose bulb was completely immersed in the esters. Heating of the flask was effected by means of an air bath constructed so as to maintain any given temperature within  $\pm 3^\circ$ . When a sample was to be removed for analysis, nitrogen was forced down the condenser tube until part of the esters was forced down the gas tube and up into a small vial. When the sample was removed, the nitrogen stream was reversed and the gas once more entered the solution through the bottom inlet of the flask.

Fig. 1 summarizes the data on the iodine numbers obtained on the esters heated at the three temperatures.

It is seen that more than 7 hours are necessary before there is any decrease in iodine number in the esters heated at 150° or 200°. At 250°, however, the iodine number drops over 20 units within 1½ hours. Also, 25 hours heating at 150° results in only a 4 unit decrease; a similar time at 200° yields approximately a 12 unit decrease, and at 250° a 62 unit decrease. Saponification equivalents determined on the esters heated at 250° were found to increase gradually with time, reaching a maximum at 68 hours after a 14 unit increase.

Since the esters heated for 68 hours at 250° appeared to be rather extensively polymerized, they were distilled at 1 mm. from a Claisen flask.

The bath temperature was allowed to reach 240° at the end, but approximately one-third of the total esters remained as a dark and viscous residue. Iodine values and saponification equivalents of the distillates increased

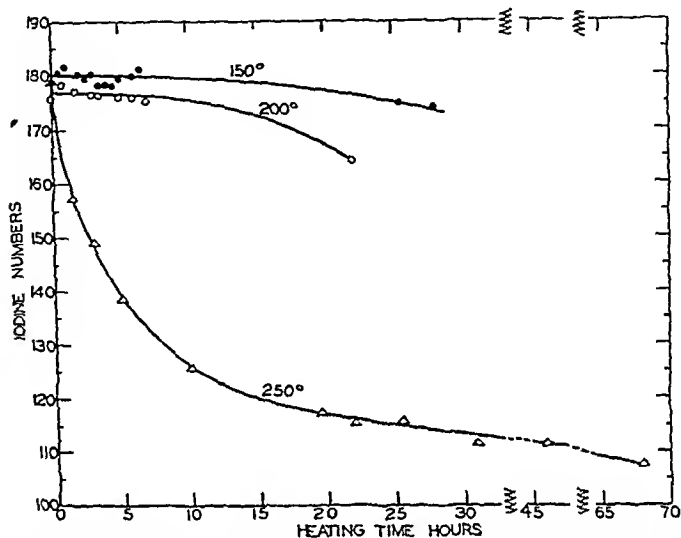


FIG. 1. Effect of time and temperature on the iodine numbers of unsaturated esters of cod liver oil.

TABLE I

*Distilled Esters of Cod Liver Oil Previously Maintained at 250° for 68 Hours*

Fraction No.	B.p. range °C.	Iodine No. (Wijs-Hg(OAc) <sub>2</sub> (3))	Saponification equivalent
1	137-147	81.2	276.4
2	153	93.2	299.4
3	155-160	105.3	320.6
4	160-174	124.5	343.7
5	178-200	128.2	382.4
Residue	Non-distillable	137.2	366.0

with the boiling point, as shown in Table I. It is noteworthy that a considerable amount of Fraction 5, possessing a saponification equivalent indicative of more than a 24-carbon ester, was obtained. In view of Hilditch's data showing that there is less than 1 per cent of 24-carbon acids in cod liver oil, the high saponification equivalent might be accounted for



by assuming the fraction to contain volatile methyl ester polymers such as described by Bradley and Johnston (4). The iodine value of the residue is subject to errors arising from substitution reactions (5). We have observed this before on other polymerized oils.

Spectroscopic data on the esters heated at the three temperatures are presented in Fig. 2. Here only the maxima at 2350 and 2700 Å. are given in order to conserve space. Absorption at 2350 Å. is usually taken as a measure of two double bond conjugation, and absorption at 2700 Å. as a measure of three double bond conjugation.

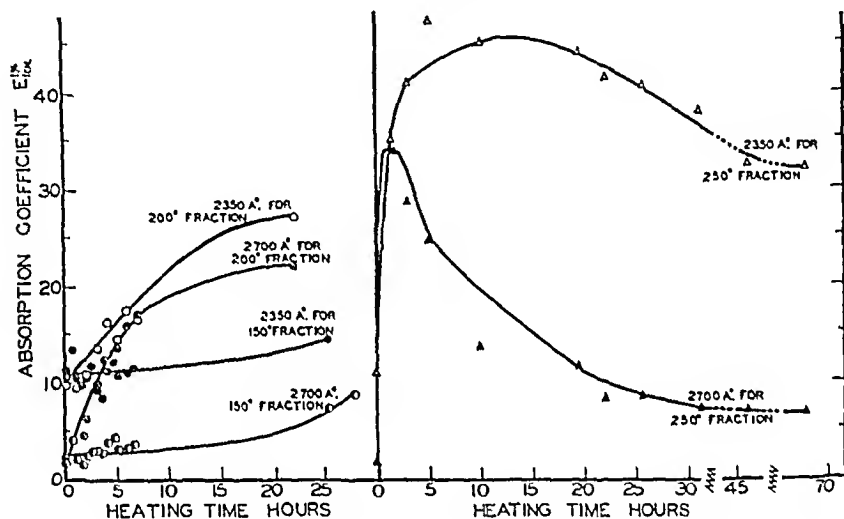


FIG. 2. Effect of time and temperature on the absorption coefficients of unsaturated esters of cod liver oil.

It is seen that heating at 150° produces no measurable increase in absorption within 25 hours, whereas heating at 200° causes detectable increase in absorption within 6 hours. In both cases, however, the amount of conjugation calculated from the absorption data is negligible. At 250° there is a distinct increase in absorption even after 1½ hours heating time, and after about 5 hours this absorption begins to decrease, indicating the removal of absorbing material. This is in agreement with the recent findings of Bradley and Johnston (4) which are advanced as additional evidence for Scheiber's hypothesis that fatty acids conjugate before polymerizing.

As a more critical test of the Scheiber hypothesis, unsaturated esters of cod liver oil were subjected to prolonged heating at 250° and absorption curves run on the residue remaining after removal of the unpolymerized

material by high vacuum distillation. As was expected, prolonged heat treatment produced a polymerized residue which possessed a high general absorption but lacked any of the maxima characteristic of conjugated double bonds. This absorption, then, would appear to be the general absorption common to cyclized molecules, and the decreased conjugate absorption accompanying polymerization must represent the deconjugation process by which polymerization is effected.

*Preparation and Distillation of Unsaturated Methyl Esters of Linseed Oil*—Unsaturated acids of linseed oil prepared by cooling a 10 per cent solution of the total acids in acetone to  $-40^{\circ}$  were esterified, yielding unsaturated esters of iodine number 189.0. These esters were distilled through a packed fractionating column (1). Chemical and physical constants determined on the original esters and on the distillates and residue are recorded in Table II.

The only absorption maxima observed were at  $2350 \text{ \AA.}$ , and here absorption may be considered negligible except in the residue. In the case of the latter the low iodine number and high absorption would appear to indicate rather extensive chemical changes. A comparable distillation of methyl linolenate, however, yields a residue with much less absorption, thus leaving unexplained the apparently anomalous behavior of the residue from the linseed oil esters.

From the lack of any absorption maxima at  $2700 \text{ \AA.}$  we must conclude that the linolenic acid of linseed oil is either entirely unchanged as a result of the heat treatment or else, and more likely, only one double bond shifts, producing a doubly conjugated system.

To obtain some idea of the total amount of conjugation produced in the distillation of the linseed oil esters the weight of each fraction was multiplied by the  $E_{1\text{cm.}}^{1\%}$  of that fraction and the total of the resulting "absorption indices" compared with the absorption index of the original esters. Thus, it was found that approximately a one-third increase in total absorption resulted from the distillation. Since the original absorption was very low, this increase is negligible.

*Preparation and Fractional Distillation of Methyl Linolenate*—The ester was prepared by the debromination of hexabromostearic acid (m.p.  $181^{\circ}$ ) according to the method of Kimura (6), and the isolated ester was used for fractional distillation without any further purification. The chemical and physical constants obtained are recorded in Table III.

A comparison of the absorption indices reveals no increase in total absorption due to distillation, although the fractionating effect of the column is illustrated by the concentration of absorptive material in the residue and highest boiling fractions.

The lack of any absorption maxima at 2700 Å. may be taken as proof that only one of the double bonds in methyl linolenate shifts so as to produce a doubly but not a triply conjugated system. It is also note-

TABLE II

*Chemical and Physical Constants of Unsaturated Linseed Methyl Ester Fractions*

Fraction No.	Heating time	B.p. at 1 mm.	Iodine No. (Wijs-Hg-(OAc) <sub>h</sub> )	Saponification equivalent	Weight of fraction	$E_{1\text{ cm.}}^{1\%}$ at 2350 Å.	Absorption index* at 2350 Å.
	min.	°C.			gm.		
Original esters			189.0		125.4	5.5	690.0
1	64	149-156	160.0	284.0	1.732	5.0	8.7
2	129	156-157	175.3	287.0	22.872	3.5	80.2
3	189	157	184.9	288.4	24.972	4.4	109.8
4	203	158	192.6	287.3	26.302	5.1	134.2
5	270	158-159	196.3	287.6	28.301	7.6	215.0
6	306	159	196.5	289.2	14.415	15.2	219.3
7 (Residue)			143.9		1.961	86.5	169.5
Total .....					120.555		936.7

\* Absorption index =  $E_{1\text{ cm.}}^{1\%}$  of fraction  $\times$  weight of fraction.

TABLE III

*Physical and Chemical Constants of Methyl Linolenate Fractions*

Fraction No.	Heating time	B.p. at 1 mm.	Iodine No. (Wijs-Hg-(OAc) <sub>h</sub> )	Saponification equivalent	Weight of fraction	$E_{1\text{ cm.}}^{1\%}$ at 2350 Å.	Absorption index at 2350 Å.
	min.	°C.			gm.		
Original esters			256.8		21.098	5.0	105.5
1	73	147-150	256.2		0.702	3.1	2.2
2	88	151-152	256.0		2.676	2.7	7.2
3	108	151-152	258.0	289.2	2.744	2.9	8.0
4	148	151	258.6	290.1	7.694	3.8	29.3
5	193	150	258.2	289.6	4.327	5.6	24.1
6	200	150	258.0		0.972	7.3	7.1
7 (Residue)					0.474	30.0	14.2
Total .....					19.598		92.1

worthy that in this case the absorption of the residue is much less than that of the linseed ester residue, as pointed out earlier.

Fractions 3, 4, and 5 possessed an iodine number approximately 99.1 per cent of theoretical as well as a saponification equivalent 99.1 per cent of theoretical, and data from other similar distillations reveal fractions of

theoretical saponification equivalent with iodine numbers over 99.1 per cent of theoretical. This is contradictory to McCutcheon's recent report (7) that methyl linolenate has an iodine number only 98.8 per cent of theoretical.

#### DISCUSSION

When unsaturated fatty acids or their derivatives are heated, there is some rearrangement of isolated to conjugated double bonds, the extent of which depends upon the degree of unsaturation of the fatty acid moiety as well as the time and temperature involved. Apparently all of the double bonds are not equally affected, since spectroscopic observations on heat-treated highly unsaturated acids from cod liver oil indicate only three double bond conjugation and purified methyl linolenate only the two double bond type.

Conjugation is only one step in the polymerization process, however. The highly reactive, conjugated double bonds undergo further change, probably by a Diels-Alder type reaction, to produce polymers devoid of conjugation. Added reagents and the nature as well as the presence or absence of solvent are probably important in this connection, since apparently a much greater concentration of conjugated material can be produced by high temperature saponification than by heating alone at the same temperature (8).

It follows, then, that a measurement of the amount of conjugation in a thermally polymerized oil can reveal only the concentration of conjugated material existing at the time of measurement without giving any hint as to the amount of material which may have passed beyond the conjugation stage. In the ordinary vacuum fractional distillation, of course, the quantity of residue left in the pot probably represents the maximum amount of material that may be polymerized, since it alone is non-volatile. Prolonged heating at high temperatures (300°), however, may produce volatile polymers in some cases (4).

Scheiber's theory (2) that isomerization of non-conjugated to conjugated double bonds is a prerequisite for heat polymerization is thus seen to be entirely consistent with recent experimental work. It accounts for the spectroscopic observation of conjugation, the increased refractive index, the splitting of iodine from Wijs solution, and the ability of heat-treated unsaturated fatty acids to add maleic anhydride under the conditions of the diene number determination. Furthermore, it is consistent with our observation that iodine numbers determined with 0.4 *N* Hanus solution (reported to react quantitatively with conjugated double bonds (9)) decrease more slowly than those determined with the standard Hanus solution which reacts incompletely with conjugated double bonds.

## SUMMARY

Fatty acids containing up to three double bonds are fairly resistant to the heat treatment involved in a vacuum fractional distillation. The more unsaturated types are less resistant to heat, the effect varying with time and temperature.

Only two double bond conjugation was observed in heat-treated methyl linolenate, and only three double bond conjugation in the more unsaturated esters of cod liver oil.

As thermal polymerization proceeds, conjugation first increases and then diminishes, the decrease paralleling the increased polymerization observed. Polymers freed from all but traces of monomers exhibit only general absorption, probably resulting from cyclization. This is in agreement with Scheiber's hypothesis that polymerization occurs through some deconjugation process.

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# A TITRIMETRIC METHOD FOR THE ESTIMATION OF SMALL AMOUNTS OF GLUCOSE

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In our studies on the effect of a toxic fraction, obtained from *Salmonella enteritidis* (1), on the blood sugar level of mice, we were faced with the problem of determining the blood sugar in as little as 0.01 ml. of blood. A survey of the literature showed that many excellent methods were available for the determination of glucose with 0.1 ml. of blood, but with the minimal quantities of blood which were at our disposal only the method of Kirk (2) was available. At that time it was felt that the special equipment and technique required for this method made it undesirable for our purpose. A study was therefore undertaken to develop a method which would permit the quantitative estimation of glucose in as little as 0.01 ml. of blood, with only the equipment generally found in a biochemical laboratory.

In recent years various modifications of the Folin and Malmros (3) colorimetric method for the estimation of glucose in blood have been published (4-7). We have carefully investigated this method, hoping to adapt it to the quantity of blood available in our studies. The results obtained, however, were not reproducible from day to day. This is in agreement with the findings of Saifer *et al.* (4) and of Klendshoj and Hubbard (5). These authors did obtain reproducible results, however, when sodium cyanide was omitted from the alkaline ferricyanide reagent and Duponol was substituted for gum ghatti as the stabilizer of the Prussian blue. Jeghers and Myers (6) as well as Reinecke (7), on the other hand, report good reproducibility, using sodium cyanide as well as gum ghatti in their respective methods, which also are adaptations of the Folin and Malmros method to smaller amounts of blood.

The potentiometric method for the estimation of glucose was first demonstrated by Shaffer in 1929 (8). The same method was used independently by Wood (9) in a physicochemical study of the reducing action of glucose. The theoretical aspects and the technique of this method are adequately discussed by Wood (9) and by Shaffer and Williams (10) and for that reason will not be repeated here. Suffice it to say that the advantages of this method make it a very useful tool in studying the reaction between alkaline ferricyanide and glucose but the disadvantages of this method limit its usefulness as a method for routine blood sugar deter-

minations. In the present study repeated use was made of the potentiometric method, particularly in investigating the effect of cyanide upon the reduction of ferrieyanide by glucose.

When the amount of ferrieyanide reduced by glucose in blood sugar determinations is estimated by titration, it is either done indirectly as in the Hagedorn and Jensen method (11) or directly as in the cerimetric ferrieyanide methods of Miller and Van Slyke (12) and of Heck, Brown, and Kirk (2). Of the two types of methods the latter seemed to be more adaptable to small amounts of glucose than the former, because it permitted the use of relatively high concentrations of ferrieyanide in the reagent. This is highly desirable, since not only is the range of glucose concentrations increased over which the reagent will give a linear response, but the sensitivity of the reagent also is increased (9). Furthermore, the well known properties of ceric sulfate make it a particularly useful reagent for carrying out oxidations in very dilute solutions.

In cerimetric titrations the end-point is usually detected by the use of an oxidimetric indicator; thus Heck *et al.* use *o*-phenanthroline, and Miller and Van Slyke use setopaline C, as indicator. Where applicable, the use of an oxidimetric indicator is the most convenient method of detecting the equivalence point. When one is working with extremely dilute solutions of ferrocyanide in the presence of relatively high concentrations of highly colored ferrieyanide, however, the addition of a quantity of indicator sufficient to produce a noticeable color may introduce a significant indicator error, and, at best, an undesirably large blank. It is not necessary, however, to use a chemical indicator in oxidimetric titrations. The equivalence point can be determined in several other ways. Among these the classical potentiometric titration method gives very accurate results, but unfortunately the time required to carry out a single determination is so long as to make the method unsuitable for use in routine glucose determinations. The elegant differential titration method of MacInnes and Jones (13) did not work satisfactorily in our hands with the extremely dilute solutions which were encountered. Müller's method of titration to the equivalence point (14), on the other hand, proved to be quite satisfactory. It is the purpose of this report to outline the points concerning the application of this method to the quantitative estimation of as small amounts of glucose as one would find in 0.01 ml. of blood.

When a solution, containing both ferrieyanide and ferrocyanide in concentrations which will be encountered in a blood sugar determination, is titrated electrometrically with ceric sulfate and the E.M.F. is plotted against the ml. of ceric sulfate added, a curve such as Curve 1, Fig. 1, is obtained. In Curve 2, Fig. 1, the same data are plotted with ml. of ceric sulfate added as abscissa and the quotient, change in E.M.F. per 0.01 ml. of ceric sulfate

added, as ordinate. The equivalence point is at the peak of the curve, where the quotient is largest.

Since in routine titrations one is solely concerned with finding the equivalence point, the actual potentials during the titration being of secondary value, Müller (14) has modified the titration in the following manner. The potential of the equivalence point for the particular oxidimetric titration having been determined empirically, an E.M.F. equal to this potential is switched into the circuit. The titrating reagent is added in small incre-

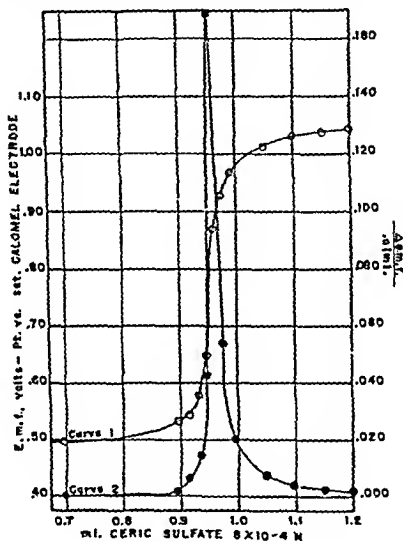


FIG. 1. Potentiometric titration of 1 ml. of  $K_3Fe(CN)_6$ , approximately  $8 \times 10^{-4} M$ , in the presence of  $K_2Fe(CN)_6$ , approximately  $2.5 \times 10^{-3} M$ , with  $Ce(SO_4)_2$ ,  $8.48 \times 10^{-4} M$ . Curve 1 shows the change of E.M.F. in the critical part of the titration. Curve 2 shows the change in the quotient E.M.F. per 0.01 ml. of  $Ce(SO_4)_2$  added during the same part of the titration.

ments and after each addition the circuit is momentarily closed by means of the usual tap key. When after the addition of a small amount of the titrating agent the galvanometer no longer shows any deflection, the end-point of the titration has been reached, for now the potential of the cell is equal to that of the equivalence point of the system. By means of this simple modification the tedious balancing of the circuit is dispensed with, as is the time-consuming plotting of results.

Objections have been raised against the Müller method of titrating to the equivalence point (14). Thus it is pointed out that polarization of the



electrode may occur during the titration. If, however, a sufficiently high resistance is placed in series with the galvanometer or if a well constructed vacuum tube, null point instrument is used, there is no need for polarization to occur. Another objection which has been raised against this method is that often the potential does not become constant immediately after the addition of the titrating agent. It was found, however, during hundreds of titrations of ferrocyanide with ceric sulfate that the potential became constant instantly, except in the immediate proximity of the equivalence point. While these objections may influence the results when the highest precision is to be desired, it is doubtful whether they influence the results of blood sugar determinations significantly, since other unavoidable errors will be considerably larger.

#### EXPERIMENTAL

##### *Reagents—*

Potassium ferrieyanide solution. Dissolve 0.8231 gm. of potassium ferrieyanide of highest purity in water and dilute to 500 ml. If stored in brown glass-stoppered bottles, it will keep for at least a week.

Sodium carbonate-chloride solution. Dissolve 17.5 gm. of sodium chloride and 31.8 gm. of anhydrous sodium carbonate in water and dilute to 500 ml. For use, mix equal volumes of the ferrieyanide solution and the sodium carbonate-chloride solution. This alkaline ferrieyanide reagent should not be kept longer than a day.

Ceric sulfate solutions. 6.0 gm. of anhydrous  $\text{Ce}(\text{HSO}_4)_4$  are dissolved in 0.5 N sulfuric acid to make 500 ml. The solution is accurately standardized and diluted to a 0.02 N solution. If kept in a glass-stoppered bottle, it will keep its titer for at least 3 months.

For use, dilute 2.0 ml. of the above stock solution and 4 ml. of 1:1 sulfuric acid to 100 ml. The resulting solution will be  $4.0 \times 10^{-4}$  N in ceric sulfate. It should be prepared fresh twice a day.

Copper sulfate solutions. Prepare a stock solution by dissolving 0.70 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in water and diluting to 100 ml. For use, dilute 5 ml. of the stock solution to 100 ml. with water.

Sodium tungstate solutions. Prepare a stock solution by dissolving 10 gm. of  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  in water and diluting to 100 ml. For use, dilute 2 ml. of the stock solution to 100 ml. with water.

##### *Procedure*

*Deproteinization*—The proteins can be removed from whole blood by means of tungstic acid, as described by Reinecke (7), 2.5 ml. of the dilute tungstic acid and 0.01 ml. of blood being used. The method of Shaffer and Williams (10) may also be employed, again half quantities of both

blood and precipitants being used. An adaptation of the Somogyi copper sulfate and sodium tungstate method (15) was found to be quite suitable.

Place 2 ml. of the dilute copper sulfate solution in a centrifuge tube. The blood, measured in a capillary pipette, calibrated to contain 0.01 ml., is added to the copper sulfate solution, and the pipette rinsed several times by drawing the solution into the pipette and blowing it out again. Next 0.5 ml. of the dilute tungstic acid solution is added. The contents of the tube are well mixed and then centrifuged.

In thin walled shell vials, 70 × 21 mm., place 2 ml. of the "filtrate" or 1 ml. of the "filtrate" and 1 ml. of water. To each vial add 1 ml. of the alkaline ferricyanide reagent. Mix the contents of the vial thoroughly and close the mouth of the vial with a glass bulb or marble. At the same time a number of blanks are prepared by substituting water for the "filtrate." All vials are placed in a wire basket and spaced about  $\frac{1}{4}$  inch apart. The basket is then placed in a vigorously boiling water bath for 10 minutes. At the end of the heating period the vials are placed in a container of cold tap water for about 3 to 5 minutes. Then 1 ml. of 15 per cent sulfuric acid is added to each vial.

The vial is clamped in a titration stand and a platinum electrode, a motor-driven glass stirring rod, the modified tip of a 2 ml. Koch micro burette (16), and the side arm of a saturated calomel half cell are introduced into the vial. Wires lead from the cell to the usual potentiometer-galvanometer hook-up with the exception that a S.P.S.T. switch is placed in series with the galvanometer. A 0.3 megohm resistance is shunted across the terminals of the switch, so that if it is thrown into the "off" position the resistance is in series with the galvanometer. By throwing it into the "on" position the resistance is removed from the circuit. The latter position is used only in the close proximity of the equivalence point. The type of instruments actually used was a type K Leeds and Northrup potentiometer and the familiar enclosed lamp and scale type galvanometer. These instruments were used simply because they were available; other types of instruments may work as well and some will work more satisfactorily. The sole purpose of the potentiometer is to furnish a current of 0.7 volt, the potential of the system at the equivalence point. Any other source of an E.M.F. which will furnish this potential can replace the potentiometer.

The titration is carried out in the following manner. The stirrer is started and a small increment of the dilute ceric sulfate is allowed to run into the solution in the vial. The tap key is momentarily depressed and the magnitude of the deflection of the galvanometer noted. With very little practice one is able to judge from this deflection whether or not the next increment of ceric sulfate should be larger or smaller than the first.

TABLE I

*Analyses of Defibrinated Beef Blood by Proposed Method*

Blood No.	Method of protein pptn.	Method of analysis	Blood sugar mg. per cent	Glucose added mg. per cent	Total glucose found mg. per cent	Total glucose expected mg. per cent
1*	Tungstic acid	Proposed	111.0			
		Folin-Wu	101.0			
2*	Zinc hydroxide	Proposed	78.8			
		Folin-Wu	81.2			
3*	Copper sulfate-sodium tungstate	Proposed	69.3			
		Folin-Wu	71.6			
4	Copper sulfate-sodium tungstate	Proposed	44.5 42.0 44.6 42.6 41.2 43.2	100	147.6 142.1 144.0 140.1 140.0 139.2	143.0
Average.....			43.0		142.1	
5	Copper sulfate-sodium tungstate	Proposed	74.8 74.9 77.0 76.2	200	269.0 269.0 269.0 268.2	275.7
Average.....			75.7		268.8	
6	Copper sulfate-sodium tungstate	Proposed	78.5 78.1 78.5 79.3 78.5	120.7	199.0 197.0 197.2 199.0 197.0	199.3
Average.....			78.6		197.8	
7†	Copper sulfate	Proposed	79.4 79.4 78.2 78.8 78.4	120.7	198.9 199.0 195.2 198.2 197.0	199.5
Average.....			78.8		197.6	

\* The values given represent the averages of six determinations.

† The samples of Blood 7 were the corresponding parts of Blood 6 which had been kept in the freezing compartment of the refrigerator for 18 hours.

The titration is thus continued by adding small amounts of reagent and tapping the key after each addition. At the equivalence point the galvanometer will show no deflection or a slight deflection in the opposite direction.

The blanks are titrated in exactly the same manner, except that the first increment of ceric sulfate added should be quite small. Subtracting the average blank titration from the titration of the sample will give the net titration from which the amount of glucose present in the sample can be calculated. The results obtained with various samples of blood are listed in Table I.

*Calculations*—1 ml. of  $4 \times 10^{-4} N$   $Ce(SO_4)_2 \approx (4 \times 10^{-7})/K$  equivalents of glucose or  $\approx (7.2 \times 10^{-2})/K$  mg. of glucose in the sample titrated. If 0.01 ml. of blood is diluted to 2.5 ml. (actually 2.51 ml.) in removing the proteins and 2.0 ml. of the filtrate are used in the determination, then this will represent 0.8 of the 0.01 ml. of blood; therefore, the burette reading minus the blank  $\times 900/K =$  mg. per cent of glucose. If only 1.0 ml. of filtrate is used in the determination, the formula becomes the burette reading minus the blank  $\times 1800/K =$  mg. per cent of glucose.  $K$  is the glucose equivalent of the alkaline ferricyanide reagent. In this laboratory the value for  $K$  for the reagent given in this report was found to be 4.82, with a standard error of 0.104.

#### DISCUSSION

In selection of an alkaline ferricyanide reagent of suitable properties the studies of Van Slyke and Hawkins (17), Shaffer and Williams (10), and Wood (9) proved to be of great value. In agreement with these authors it was found that decreasing the alkalinity of the reagent increased the sensitivity; that is, increased the value for the glucose equivalent, but decreased alkalinity also increased the time required for the oxidation of the glucose to reach completion. The results obtained with three different reagents of varying alkalinity but constant ferricyanide content are shown in Fig. 2 to illustrate this point.

It will be noticed that in the case of Reagent 1 the reaction is completed within 3 minutes and the glucose equivalent, although the lowest for the three reagents, remains constant even with prolonged heating. The glucose equivalent of Reagent 2 is higher than that of Reagent 1, but the reagent reacts much more slowly. Heating for about 10 minutes is required before the glucose equivalent becomes constant. Reagent 3 is the most sensitive reagent, but it also reacts most slowly. When these results are evaluated, it should be borne in mind that they were obtained with relatively low concentrations of glucose, 4  $\gamma$  per ml. If the concentration

is increased, the reaction will be slowed up in all cases. This fact, often neglected by investigators, has been stressed by Wood (9). It is of particular importance in work with reagents of low alkalinity.

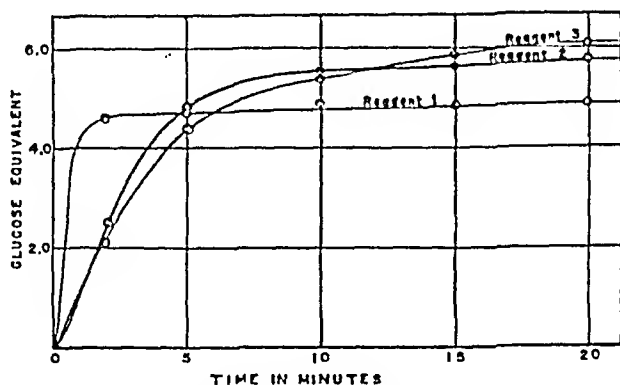


FIG. 2. Time curves showing the effect of varying the alkalinity upon the glucose equivalent of ferrieyanide reagents. Reagent 1,  $\text{Na}_2\text{CO}_3$  (0.3 M),  $\text{NaCl}$  (0.3 M); Reagent 2,  $\text{Na}_2\text{CO}_3$  (0.4 M),  $\text{NaHCO}_3$  (0.2 M); Reagent 3,  $\text{Na}_2\text{CO}_3$  (0.3 M),  $\text{NaHCO}_3$  (0.3 M). The potassium ferrieyanide concentration in all three reagents was  $2.5 \times 10^{-3}$  M. In all cases 2 ml. of a glucose solution, containing 4  $\gamma$  of glucose per ml., and 1 ml. of the alkaline ferrieyanide solutions were used.

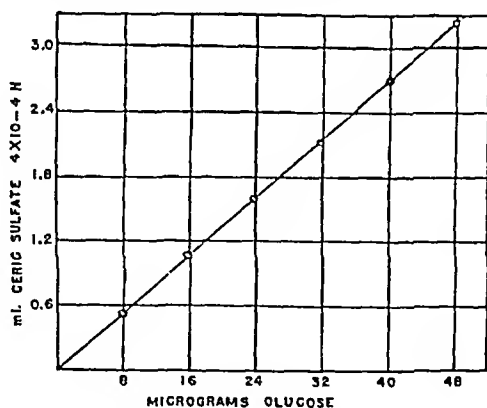


FIG. 3. Effect of varying the glucose concentration upon the alkaline ferrieyanide reagent, expressed in terms of ml. of ceric sulfate used to titrate the ferroeyanide formed. The heating time in all cases was 8 minutes. If 2 ml. of a 1:250 blood filtrate are used, 4  $\gamma$  of glucose per ml. correspond to a blood sugar value of 100 mg. per cent.

In order to determine the response of Reagent 1 to higher concentrations of glucose, varying amounts of glucose were heated with the reagent for a fixed period of 8 minutes. The results, as plotted in Fig. 3, show that under

these conditions the reagent gave a linear response with amounts of glucose as high as 48  $\gamma$  (higher concentrations of glucose were not tried). For routine analyses a heating time of 10 minutes was chosen to be quite certain that the oxidation of glucose is complete, even with the highest concentration of glucose which one might reasonably expect. In view of the fact that with Reagent 1 the heating period can be extended to 20 minutes, as shown in Fig. 2, without affecting the glucose equivalency factor, the choice of a 10 minute heating period for routine analyses is justified.

In a recent publication Reinecke (7) has described the use of an alkaline ferricyanide reagent containing sodium cyanide for the determination of glucose in very small amounts of blood. Wood (9) has investigated the effect of cyanide upon the oxidation of glucose by ferricyanide and found that the addition of cyanide increases the glucose equivalent of the reagent and that this increase in reducing action shown by glucose is not shown by non-glucose reducing substances found in tungstic acid filtrates. Yet he cautions against the use of cyanide in an alkaline ferricyanide reagent until further studies have been made, because in the presence of cyanide the glucose equivalent of an alkaline ferricyanide reagent varies with the ratio of the cyanide to glucose concentration. Saifer *et al.* (4) also have investigated the use of cyanide in alkaline ferricyanide reagents for the determination of blood sugars. They found that the inclusion of cyanide in the reagent affected the reproducibility of the results.

In the present investigation the effect of cyanide upon the glucose equivalent was also studied, the potentiometric method of Shaffer and Williams (10) being used. When reagents of the same composition and the same concentration as those employed by Wood (9) were used, his results were confirmed in all respects. However, when much lower concentrations of alkaline ferricyanide and correspondingly lower concentrations of glucose and cyanide were used, it was found that the addition of cyanide did not change the glucose equivalent of the reagent, even when the ratio of moles of cyanide to moles of glucose was 10, a ratio which gave the maximum increase with the more concentrated reagents. When the amount of ferrocyanide formed in the oxidation of glucose was estimated colorimetrically by an adaptation of the Folin and Malmros method (3), the results were not reproducible. Whether or not this was due to the sodium cyanide or to the gum ghatti used was not investigated. In order to conserve space, the protocols dealing with the potentiometric and colorimetric studies on the effect of cyanide are not included in this report.

#### SUMMARY

A cerimetric titration method for the estimation of small amounts of glucose has been described.

It was found that the Möller principle of titrating to the equivalence point is a convenient method of detecting the end-point in the case of very dilute solutions of potassium ferrocyanide and ceric sulfate.

With tungstic acid filtrates the proposed method gives somewhat higher blood sugar values than the Folin-Wu method. With zinc hydroxide and with copper sulfate-sodium tungstate filtrates it gives about the same values as does the Folin-Wu method with these filtrates.

The recovery of glucose added to blood is 98.5 per cent.

The author takes this opportunity to express his gratitude for a Grant-in-Aid from the American Academy of Arts and Sciences, Permanent Science Fund, which in part has supported the author's studies on the biochemistry of a toxin prepared from *Salmonella enteritidis*. The present report deals with a part of these studies.

The author is also greatly indebted to Professor W. R. Tweedy for his valuable advice and his helpful criticism.

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# INFLUENCE OF LINOLEIC AND PALMITIC ACIDS OF THE DIET ON SYNTHESIS AND STORAGE OF FATTY ACIDS IN THE WHITE RAT\*

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The composition of the fat of the animal body appears to be rather characteristic of the species. The fatty acids are recognized to have several origins. They may be those of the diet, those synthesized from carbohydrates and proteins, and those formed by interconversion from other fatty acids.

The present study was directed primarily toward a consideration of this third possibility, to ascertain what fatty acids would be stored following the addition of palmitic acid to a basal ration quite low in lipids. For control the fatty acids stored by rats continued on the basal ration were determined.

Since rats require, for normal growth and normal metabolism, linoleic acid or certain other unsaturated fatty acids, it has also seemed desirable to have additional experiments in which the basal ration was supplemented not only with palmitic acid but also with linoleic acid.

Male albino rats taken shortly after weaning were fed for 45 days on a lipid-low basal diet (Diet I). This diet contained casein (extracted with fat solvents) 20, extracted yeast (Harris, fat-free) 10, salt mixture 3 (1), and sucrose 67 per cent. By analysis the casein was found to contain 0.5 per cent, the yeast 1.2 per cent, and the diet not more than 0.25 per cent of fatty acids. By the end of the period all the rats showed definite signs of the Burr and Burr syndrome, with scaly hind feet, ridged and scaly tail, waxy ears, and a reduced rate of growth.

Group A (eight rats), continued on the basal ration, consumed during 102 days about 1100 gm. of this diet each. Growth ceased after about 85 days. The average weight reached was 179 gm.

Group B (seven rats), continued on Diet II, identical with Diet I except that 5 per cent of palmitic acid replaced an equal amount of sucrose, ceased growing after about 85 days, but reached an average weight of 186 gm. The symptoms of deficiency were milder than those of the control group.

\* Based on a doctoral thesis, Purdue University, 1940.

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On an average 545 gm. of Diet II were consumed by each rat during the second period of 54 days.

Each animal in Group C (eight rats), continued on Diet II, received, until the 88th day, a weekly supplement of 3 drops (about 100 mg.) of methyl linoleate. These animals grew more rapidly than the others and continued to grow, though slowly, to the end. The average weight reached was 211 gm. The symptoms of deficiency became milder, although several rats exhibited somewhat scaly tails throughout. During the second period of 54 days, an average of 590 gm. of Diet II was ingested.

The diets of all the animals were supplemented weekly with purified fat-soluble vitamins. Vitamin A was supplied as carotene (330 i.u. per week), vitamin D as irradiated ergosterol (50 i.u. per week), and vitamin E as the unsaponifiable matter from 1 gm. of wheat germ oil per week. Sterols and traces of fatty acids were removed from the latter preparation according to the method of Mackenzie, Mackenzie, and McCollum (2).

At the end of the experimental feeding period, the rats were allowed to fast for 24 hours and then were anesthetized and sacrificed. The kidneys and livers were discarded and the remainder of each carcass prepared for extraction by grinding in a meat chopper. Abundant mesenteric fat was present.

*Extraction and Analysis of Carcass Lipids*—The carcass lipids were removed by exhaustive extraction with low boiling fat solvents. Methyl alcohol, used first, served to dehydrate the carcasses. Extraction was continued until no more than a gm. of lipids was extractable by covering the ground rats with acetone and refluxing for 3 hours. A final extraction was made with ether.

The lipid extract was carefully saponified by adding in two portions a minimum amount of potassium hydroxide, in order to avoid alteration of linoleic acid. Unsaponifiable matter was removed by ether extraction of the alkaline solution. After acidification, the fatty acids were removed by extraction with ether. The fatty acids were separated into liquid and solid fractions by the method of Twitchell (3). They were esterified by refluxing with 10 times their weight of anhydrous methyl alcohol containing 5 per cent of concentrated sulfuric acid. The esters were freed of methyl alcohol, washed with distilled water, then with dilute sodium carbonate solution, and finally with water. They were fractionated at a pressure of about 1 mm. of Hg in a 4 foot Fenske column patterned in general after that of Longenecker (4).

Iodine number determinations on the large samples were made by the method of Wijs with a halogenation period of  $\frac{1}{2}$  hour in the dark. For the smaller samples of 2 gm. or less, the method of Trappe (5) was used. The determination of the average molecular weight of the fatty acids in the

methyl esters was accomplished by direct titration of the isolated fatty acids.

*Calculation of Results*—The calculation of the percentage composition from the equivalent weights of the fatty acids and the iodine numbers of the methyl esters was accomplished by apportioning the weight of a given sample between two or three fatty acids. Methods of calculation have been described by Harper *et al.* (6) and by Channon *et al.* (7). Because of

TABLE I

*Detailed Analysis of Methyl Esters of Acids from Rats of Group A, Grown on Fat-Low Diet*

Fraction No.	Weight	Iodine No.	Equivalent weight of acid	Saturated				Unsaturated				
				C <sub>12</sub>	C <sub>14</sub>	C <sub>16</sub>	C <sub>18</sub>	C <sub>18</sub>	C <sub>18</sub>	C <sub>18</sub>	C <sub>20</sub>	Linoleic acid
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
S-1	1.22	1.32	229.4		1.17	0.05						
S-2	0.67	3.1	242.1		0.36	0.31						
S-3	23.25	2.7	257.7			21.41	1.17		0.67			
S-4	6.73	2.8	257.5			6.23	0.30		0.20			
S-5	0.79	4.6	260.4			0.64	0.11		0.04			
S-6	2.29	29.0	279.1			0.32	1.19				0.78	
S-7	1.45	24.0	283.0			0.05	0.99				0.41	
S-8	1.22	13.8	283.6			0.02	1.00				0.20	
L-1	1.07	29.7	225.9	0.06	0.66			0.35				
L-2	0.58	42.4	234.4		0.33			0.10	0.15			
L-3	14.99	82.0	255.5			1.84			12.61	0.54		
L-4	0.98	69.3	259.5			0.25			0.57	0.16		
L-5	26.27	85.9	283.0							26.17		0.10
L-6	25.11	84.8	281.9							25.06		0.05
L-7	1.56	78.9	279.0			0.15				1.41		
L-8	1.68	85.9	284.3							1.68		
L-9	3.11	113.0	298.1							1.37	1.74	
Total . .	112.97			0.06	2.52	31.27	4.76	0.45	14.24	57.78	1.74	0.15
Mole % . .				0.07	2.60	29.03	4.00	0.47	13.33	48.99	1.34	0.13

the comparatively pure fractions obtained, the following simplified method was employed. In the fractions with iodine numbers less than 40 the iodine number was ascribed to the unsaturated acid of the same chain length as the principal component of the fraction. The difference between the theoretical equivalent weight of the principal component and the observed equivalent weight was ascribed to the adjacent saturated acid with an even number of carbon atoms. In the unsaturated (liquid) acid analysis when the iodine number was greater than 40 a similar method was employed. The difference between the theoretical equivalent weight of the

principal component and the observed equivalent weight was again ascribed to the adjacent unsaturated acid with an even number of carbon atoms. Then a calculated iodine number was determined from the theoretical iodine numbers of the adjacent unsaturated acids, depending on their respective abundance in the fraction. If the observed iodine number was higher than this calculated value, the difference was ascribed to linoleic acid. If it was lower, the difference was ascribed to a saturated acid of chain length equal to that of the shorter of the two unsaturated acids. A second approximation was occasionally necessary in determining the calculated iodine number when the amount of the saturated acid was appreciable.

Table I presents data obtained from Group A. Table II presents a summary of the results obtained with all three groups.

TABLE II

*Summary of Fatty Acid Analyses in Mole Per Cent*

Group A was on a fat-low diet; Group B received 5 per cent of the diet as palmitic acid; Group C received  $\frac{1}{2}$  drop daily of linoleic acid in addition to 5 per cent of the diet as palmitic acid.

Saturated acids	Group A	Group B	Group C	Unsaturated acids	Group A	Group B	Group C
Lauric.....	0.1	0.1	0.1	Tetradecenoic ..	0.5	0.6	0.5
Myristic .....	2.6	2.2	2.5	Hexadecenoic ..	13.3	18.0	15.8
Palmitic.....	29.0	29.0	26.6	Octadecenoic ..	49.0	44.2	48.1
Stearic.....	4.0	3.9	4.2	Linoleic. ....	0.1	0.7	1.4
Arachidic.....			0.2	C <sub>20</sub> .....	1.3	0.7	1.2
Total.....	35.7	35.2	33.6	Methyl esters, gm.	113	112.7	164.5

*Analysis of Fecal Lipids*—The feces of the rats receiving palmitic acid were collected and extracted with ether and acetone to determine the lipid content. The lipids were separated into free fatty acids, esterified fatty acids, and unsaponifiable matter. The total lipids extracted from the collected feces of the rats of Group B weighed 56 gm. and contained 48 gm. of free fatty acids of average molecular weight 273 (palmitic acid = 256), and iodine number 5.8, while those from Group C weighed 35 gm. and contained 29 gm. of free fatty acids of molecular weight 272 and iodine number 5.5.

*Results*

The rats of Group A raised on a diet quite low in lipids, containing 10 per cent of brewers' yeast, synthesized moderately large amounts of fatty acids, averaging 13 gm. per rat. The rats (Group B) receiving in

addition 5 per cent of palmitic acid deposited approximately the same amount of fatty acids. The rats (Group C) receiving in further addition about 100 mg. of methyl linoleate weekly exhibited about half again as large an amount of fatty acids.

The relative amounts of the several saturated fatty acids in the three groups were essentially the same. The somewhat lower percentage of palmitic acid in Group C, while suggestive, is not now taken to have definite significance. It is interesting to note that the animals receiving palmitic acid showed more of the palmitoleic acid than the controls. Whether the differences in the values for the animals in Groups B and C are characteristic, further work will have to establish.

The results here reported provide no evidence that the addition of 5 per cent of palmitic acid to an otherwise fat-poor diet affects the relative amounts of the various fatty acids stored in the body of the white rat. While it is conceivable that the administered palmitic acid was oxidized at once, this appears improbable in the light of Schoenheimer and Rittenberg's (8) conclusion that, "the largest part of the diet fat, even when it is present in small quantities [less than 5 per cent], is deposited in the fat tissues before it is utilized." Presumably therefore the ingested palmitic acid is transformed to a mixture of fatty acids essentially the same as that synthesized on a diet low in lipids. The further addition of linoleic acid to the diet does not change the picture qualitatively, although the storage of fat was increased considerably.

#### SUMMARY

The kinds of fatty acids stored by the rat were essentially the same on a diet low in lipids as on this diet supplemented with 5 per cent palmitic acid, with or without additional linoleic acid.

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# A NOMOGRAPHIC REPRESENTATION OF CERTAIN PROPERTIES OF THE PROTEINS

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Of the various properties of the proteins that which may well be ranked first, both because of its intrinsic importance and the amount of study which it has received, is molecular weight. Moreover this basic property stands in a central and coordinating position in relation to many others. This is apparent from a consideration of the methods by which it may be determined. The principal ones are those based on (1) chemical analysis, (2) osmotic pressure, (3) dialysis with a graded series of calibrated membranes, (4) x-ray determination of the dimensions of the unit cell in the crystal, (5) sedimentation equilibrium, (6) sedimentation velocity, (7) diffusion constant, and (8) relaxation time. Method 1 yields only a minimum value of the molecular weight, but has the advantage that it is independent of other considerations and is often susceptible to great precision. Method 2 has been one of the principal sources of knowledge, but is complicated by the corrections arising in connection with the Donnan equilibrium. These involve electromotive force measurements, a knowledge of activity coefficients, and in some cases of the amphoteric properties of the protein. Method 3 gives qualitative results only, serving at best simply to place a given protein in relation to others. Method 4 provides a maximum value of the molecular weight, in conjunction with a knowledge of the density of the crystal and the water of hydration. Method 5 gives results which, after correction for the effects of diffusible ions, in case the protein is not isoelectric, are independent of all other properties of the protein save the partial specific volume. Method 6 gives results which can be interpreted in terms of molecular weight only in connection with information regarding a translational frictional ratio in addition to a knowledge of the partial specific volume. Nevertheless, it has probably yielded more information than any other method, and has the advantage of contributing additional information regarding the homogeneity of the protein. Method 7 yields information which can be interpreted in terms of molecular weight only on the basis of a knowledge of the same frictional ratio, as well as of the partial specific volume. This frictional ratio may itself be interpreted in terms of hydration and shape. As is well known, however, Methods 6 and 7 may be combined in such a way as to eliminate the troublesome frictional ratio. Method 8 yields results which, like

those based on sedimentation and diffusion, may be interpreted only with the aid of additional knowledge of a frictional ratio, this time, however, a rotational frictional ratio. This ratio may in turn also be interpreted in terms of hydration and shape.

These considerations indicate the way in which, from an operational point of view, the property of molecular weight is bound up with various other properties. Through the molecular weight, or otherwise, there are mutual implications involving all these, such that a knowledge of one is contributory to a knowledge of the others. In this study we shall attempt to exhibit certain of these interrelations by means of a particularly effective type of nomogram, an alignment chart. Specifically, we shall concern ourselves with molecular weight, sedimentation constant, diffusion constant, frictional ratio, hydration, shape, and relaxation time. In addition to these we shall introduce one other property, not immediately related to the molecular weight but closely involved with two others, hydration and shape, which are themselves more directly connected with molecular weight. This property is the viscosity increment, defined below (see Equation 12). Such a nomographic treatment serves to present in a readily comprehensible form a set of complicated relations, involving many variables, which otherwise remain difficult to visualize. In this respect it offers a more compact and legible picture of the situation than that provided by such Cartesian contour charts, valuable and illuminating as they are, as those given by Oneley (4). Moreover, it makes it possible to represent together on a single sheet, or two sheets, different experimental findings on a given protein, and to synthesize them into a consistent picture of the molecule in question.

### *Fundamental Equations*

In order to construct such a nomogram as we propose it is necessary to set down systematically the various equations involving the different properties as variables. To facilitate this we introduce at this point the following table of symbols.

$m$	= weight of a single molecule of the protein
$M$	= " " " gm. molecule of the protein
$N$	= Avogadro's number = $6.025 \times 10^{23}$
$s$	= sedimentation constant
$s_{20}$	= reduced sedimentation constant; gives the ideal value of $s$ for an infinitely dilute aqueous solution at $20^\circ$
$k$	= molecular gas constant = $1.380 \times 10^{-16}$ erg per degree
$T$	= absolute temperature
$c$	= concentration of protein in gm. per cc.
$d$	= density of the solution
$d_{20}$	= " " water at $20^\circ$ = 0.9982

- $\bar{v}$  = partial specific volume of the protein  
 $\bar{v}_{20}$  = " " " " " " " in an infinitely dilute aqueous solution at 20°  
 $h$  = water of hydration, expressed as volume fraction of the unhydrated molecule  
 $f$  = force of friction encountered by a single molecule moving with unit translational velocity through the medium  
 $f_0$  = ideal value of  $f$  calculated by treating the molecule as an unhydrated sphere  
 $D$  = diffusion constant =  $kT/f$   
 $D_{20}$  = reduced diffusion constant; gives the ideal value of  $D$  for an infinitely dilute aqueous solution at 20°  
 $r$  = radius of the protein molecule, regarded as a sphere  
 $\rho$  = ratio of the two axes of the hydrated molecule, regarded as an ellipsoid of revolution; transverse axis divided by axis of revolution  
 $\eta$  = viscosity of the solution  
 $\eta_{20}$  = " " water at 20° = 0.01009  
 $B_\eta$  = " increment. See Equation 12 below. This is the same as the quantity denoted by  $\nu$  by Simha  
 $\tau_1$  = relaxation time for a moment parallel to the axis of revolution of the molecule regarded as an ellipsoid of revolution  
 $\tau_2$  = relaxation time for a moment perpendicular to the axis of revolution  
 $\tau_0$  = ideal value of the relaxation time of the molecule regarded as an unhydrated sphere  
 $\zeta$  = rotational frictional constant  
 $\zeta_0$  = ideal rotational frictional constant of the molecule regarded as an unhydrated sphere

We begin with the basic equation involving the sedimentation constant. This may be written as

$$(1) \quad m = \frac{fs}{(1 - \bar{v}d)} = \frac{f}{f_0} \cdot f_0 \frac{s}{(1 - \bar{v}d)}$$

$f_0$  is given by the Stokes equation

$$(2) \quad f_0 = 6\pi\eta r$$

At the same time  $m$  is given by

$$(3) \quad m = \frac{4}{3} \pi r^3 \bar{v}$$

Combination of Equations 1, 2, and 3 gives

$$(4) \quad m^2 = 162\pi^2 \frac{\bar{v}}{(1 - \bar{v}d)^2} \cdot \eta^2 s^2 \left( \frac{f}{f_0} \right)^2$$

This equation may be rewritten as

$$(5) \quad N^2 m^2 = [162\pi^2 N^2 \eta_{20}^2] \frac{\bar{v}_{20}}{(1 - \bar{v}_{20}d_{20})^2} \left[ \frac{\bar{v}}{\bar{v}_{20}} \frac{(1 - \bar{v}_{20}d_{20})^2}{(1 - \bar{v}d)^2} \frac{\eta^2}{\eta_{20}^2} s^2 \right] \left( \frac{f}{f_0} \right)^2$$



If we introduce numerical values, identify  $s_{20}^3$  with the second expression in brackets (this in fact constitutes the definition of  $s_{20}$ ), and take the square root of both sides, Equation 5 becomes

$$(6) \quad M = 2.442 \times 10^{22} \frac{\bar{v}_{20}^4}{(1 - 0.9982\bar{v}_{20})^4} \cdot s_{20}^4 \left( \frac{f}{f_0} \right)^4$$

A corresponding expression may be derived for the diffusion constant  $D$ . We start with the basic equation

$$(7) \quad D = \frac{kT}{f} = \frac{kT}{f_0} \cdot \frac{f_0}{f}$$

If we combine this with Equations 2 and 3, we obtain

$$D^3 = \frac{f_0^3}{f^3} \cdot \frac{(kT)^3}{162\pi^2\eta^3 m \bar{v}}$$

or, introducing Avogadro's number and rearranging, we get

$$(8) \quad M = \left( \frac{f_0}{f} \right)^3 \cdot \frac{N^3 k^3 T_{20}^3}{162\pi^2 N^2 \eta_{20}^3 \bar{v}_{20}} \left[ \frac{\eta_{20}^3}{\eta^3} \cdot \frac{\bar{v}_{20}}{\bar{v} D^3} \cdot \frac{T^3}{T_{20}^3} \right]$$

Substituting numerical values and identifying  $1/D_{20}^3$  with the expression in brackets (this constitutes the definition of  $D_{20}$ ), we obtain

$$(9) \quad M = 2.428 \times 10^{14} \left( \frac{f_0}{f} \right)^3 \frac{1}{D_{20}^3 \bar{v}_{20}}$$

We now have the task of setting down the expression for  $f/f_0$  in terms of the hydration  $h$  and axial ratio  $\rho$ . In doing this we shall assume that the molecules may be adequately treated as ellipsoids of revolution. If we introduce  $f'_0$  to denote the ideal value of the frictional constant for the *hydrated* protein molecule, regarded as a sphere, we may write

$$(10) \quad \frac{f}{f_0} = \frac{f}{f'_0} \cdot \frac{f'_0}{f_0}$$

From Equation 2 we see that  $f'_0/f_0 = (1 + h)^{\frac{1}{3}}$ .  $f/f'_0$  may be obtained from the equations developed by Perrin (6) for prolate and oblate ellipsoids. We shall denote it by  $\varphi(\rho)$ . We write therefore as the over-all equation

$$(11) \quad \frac{f}{f_0} = (1 + h)^{\frac{1}{3}} \varphi(\rho)$$

Tables giving numerical values of  $\varphi(\rho)$  calculated from Perrin's equations for both prolate and oblate ellipsoids are given by Svedberg and Peder-  
sen ((8) p. 41).

We pass now to the viscosity increment  $B_\eta$ . This is defined in terms of

the relative viscosity  $\eta/\eta_0$ ,  $\eta$  denoting the viscosity of the solution,  $\eta_0$  that of the solvent, by

$$(12) \quad B_\eta = \left( \frac{\eta}{\eta_0} - 1 \right) / c\bar{v}$$

It is thus directly determinable by experiment, but the significant value of  $B_\eta$  is that extrapolated to infinite dilution. Now the value of  $(\eta/\eta_0 - 1)$  can be shown theoretically to depend only on the shape of the dissolved particles and the volume fraction  $c'$  which they occupy in solution.

$$(13) \quad \left( \frac{\eta}{\eta_{H_2O}} - 1 \right) = \psi \cdot c'$$

where  $\psi$  is a function of the shape. If we introduce the hydration  $h$  (expressed as a volume fraction)

$$(14) \quad c' = c\bar{v}(1 + h)$$

and the equation becomes

$$(15) \quad \left( \frac{\eta}{\eta_0} - 1 \right) = \psi \cdot c\bar{v}(1 + h)$$

For spheres, Einstein has calculated the value of  $\psi$  as 2.5. Other investigators have dealt with the value of  $\psi$  for ellipsoids. In particular Simha has given equations which express  $\psi$  as a function of  $\rho$  for ellipsoids of revolution, both oblate and prolate. If we treat the molecules as ellipsoids of revolution, therefore, we can write

$$(16) \quad B_\eta = (1 + h) \cdot \psi(\rho)$$

Tables giving values of  $\psi(\rho)$  calculated from Simha's equations are now available (3).

Finally we turn to the equations for the relaxation times. For a spherical particle the relaxation time is defined in terms of the rotational frictional constant  $\zeta$  by the relation

$$(17) \quad \tau = \zeta / 2kT$$

$\zeta$  is given by Stokes as

$$(18) \quad \zeta = 8\pi\eta r^2$$

and is seen to be proportional to the volume of the sphere.  $\tau$  is a measure of the time required for a set of spherical particles initially lined up as regards some fixed direction in each to revert to a random distribution. In the case of a set of spherical molecules having an electric moment, the relaxation time gives rise to the phenomenon of dielectric dispersion involving a single critical frequency. It may be obtained from determinations of this frequency.

For bodies other than spheres the situation is more complicated. The frictional coefficient depends on the axis about which the rotation takes place and it is no longer possible to speak of a single relaxation time. The case of an ellipsoidal particle has been treated in detail by Perrin (5). For an ellipsoid there are three relaxation times, one corresponding with each of the three principal axes. Each of these is given by an expression of the same form as that for the single relaxation time of a sphere; e.g.,  $\tau_1 = \zeta_{23}/2kT$ . The meaning of the subscripts requires explanation.  $\tau_1$  refers to the orientation of axis 1, and  $\zeta_{23}$  is the harmonic mean of the frictional coefficients for rotations about the two other principal axes. Corresponding to each of these three relaxation times there will be a critical frequency in the case of polar ellipsoidal molecules whose electric moment has a component along each of the three axes.

In the case of ellipsoids of revolution, two of the frictional coefficients, and consequently two of the relaxation times, become identical. Perrin discusses this case at length and evaluates the ratio of each of the two frictional coefficients to that of a sphere having the same volume as the ellipsoid. These two ratios are given as functions of the axial ratio  $\rho$  of the ellipsoid (length of the transverse axis divided by the length of the axis of revolution). From them he at once obtains the two ratios  $\tau_1/\tau_0$  and  $\tau_2/\tau_0$  which express respectively the relaxation times for moments parallel and perpendicular to the axis of revolution divided by the relaxation time of a sphere of equal volume.

If we treat the protein molecules as ellipsoids of revolution, we can make use of these results of Perrin to express the two relaxation times in terms of molecular weight, axial ratio, and hydration. To do this we write

$$(19) \quad \tau_1 = \frac{\tau_1}{\tau'_0} \cdot \frac{\tau'_0}{\tau_0} \cdot \tau_0$$

introducing  $\tau'_0$  to denote the ideal relaxation time of the *hydrated* molecule regarded as a sphere, and  $\tau_0$  that of the *unhydrated* molecule regarded as a sphere. From Equation 18 it follows that  $\tau'_0/\tau_0 = 1 + h$  and from Equations 3 and 17 and 18

$$(20) \quad \tau_0 = \frac{3\eta m \bar{v}}{kT}$$

$\tau_1/\tau'_0$  is given as a function of  $\rho$  by the results of Perrin.<sup>1</sup> We shall denote it by  $\chi(\rho)$ ; therefore,

$$(21) \quad \tau_1 = \chi_1(\rho)(1 + h)3\eta \frac{M\bar{v}}{NkT}$$

<sup>1</sup> There is a misprint in Perrin's paper (5). In the second of Equations 95 the expression  $(2b^2 - a^2)$  in the denominator on the right should be  $(a^2 - 2b^2)$ . In the second of Equations 96 and of 96 bis the corresponding expression  $(2\rho^2 - 1)$  should be  $(1 - 2\rho^2)$ .

As in the case of sedimentation and diffusion constants it is desirable to use a reduced value of the relaxation time. For this purpose we rewrite Equation 21 as

$$(22) \quad (\tau_1)_{20} = \tau_1 \frac{\eta_{20} \bar{v}_{20}}{\eta \bar{v}} \frac{T}{T_{20}} = \frac{3\eta_{20} M \bar{v}_{20}}{NkT_{20}} \chi_1(\rho)(1+h)$$

Then, introducing numerical values, we obtain finally

$$(22') \quad (\tau_1)_{20} = 1.2421 \times 10^{-12} \bar{v}_{20} M \chi_1(\rho)(1+h)$$

The same expression applies to  $\tau_2$  with a change of subscript

$$(23) \quad (\tau_2)_{20} = 1.2421 \times 10^{-12} \bar{v}_{20} M \chi_2(\rho)(1+h)$$

According to Perrin we adopt the convention that  $\tau_1$  refers to the relaxation time for a moment parallel to the axis of revolution, and  $\tau_2$  to that for a moment perpendicular to the axis of revolution.

### Construction of Nomograms

The equations that we are concerned with in the construction of the nomogram are Nos. 6, 9, 11, 16, 22', and 23. These may be rewritten in logarithmic form as follows:

$$(24) \quad \log M - \left( 22.3877 + \log \frac{(\bar{v}_{20})^{\frac{1}{2}}}{(1 - 0.9982\bar{v}_{20})^{\frac{1}{2}}} + \frac{3}{2} \log s_{20} \right) - \frac{3}{2} \log \frac{f}{f_0} = 0$$

$$(25) \quad \log M + (13.6148 + 3 \log D_{20} + \log \bar{v}_{20}) + 3 \log \frac{f}{f_0} = 0$$

$$(26) \quad \log \frac{f}{f_0} - \frac{1}{3} \log (1+h) - \log \varphi(\rho) = 0$$

$$(27) \quad \log B_\eta - \log (1+h) - \log \psi(\rho) = 0$$

$$(28) \quad (\log (\tau_1)_{20} - \log \bar{v}_{20} + 11.9059) - \log M - \log (1+h) - \log \chi_1(\rho) = 0$$

$$(29) \quad (\log (\tau_2)_{20} - \log \bar{v}_{20} + 11.9059) - \log M - \log (1+h) - \log \chi_2(\rho) = 0$$

There are six of them and it will be seen that they contain ten variables:  $s_{20}$ ,  $D_{20}$ ,  $M$ ,  $f/f_0$ ,  $\bar{v}_{20}$ ,  $B_\eta$ ,  $(\tau_1)_{20}$ ,  $(\tau_2)_{20}$ ,  $h$ ,  $\rho$ . A knowledge of any four of these variables therefore serves to determine the remaining six, subject to the applicability of the equations. Of these ten variables seven, namely  $s_{20}$ ,  $D_{20}$ ,  $M$ ,  $\bar{v}_{20}$ ,  $B_\eta$ ,  $(\tau_1)_{20}$ ,  $(\tau_2)_{20}$ , are capable of experimental determination; and, except for the fact that the partial specific volume is involved in the other six, they are capable of independent determination, for the molecular weight, which is the only apparent exception, may be estimated independently of the rest from a combination of osmotic pressure measurements, chemical analysis, and x-ray studies. Of the remaining three variables,

$h$  is susceptible to estimation from experiment but is in general not so accurately measurable as the others.  $\rho$  and  $f/f_0$  are not determinable except in terms of the other variables on the basis of the above equations. From all this it is clear that questions of consistency are certain to arise for proteins for which many experimental data are available and that the most reliable and complete picture of the characteristics of the molecule will be obtained by interrelating the results. As we have said, a nomographic representation of the situation helps greatly to settle questions of consistency and to present in a comprehensible way the somewhat complex relationships involved.

Consideration of the six equations in logarithmic form shows that they are all linear in functions of each of the ten variables. This renders them particularly well suited to representation in terms of an alignment chart. Let us begin with Equations 24 and 26. These involve six of the variables, but if we associate two of them by introducing a parameter we reduce the number to five. These may then be represented by a simple type of double alignment chart, in which the scales are all straight parallel lines, the pivot scale being also the scale for one of the variables. Such a double alignment chart is in reality nothing but a combination of two single alignment charts, each involving three variables, which have one scale in common. In order to carry this into effect we treat the expression in parentheses in Equation 24 as a new variable,  $\sigma$ . Then Equation 24 becomes

$$(30) \quad \log M - \sigma - \frac{3}{2} \log \frac{f}{f_0} = 0$$

This equation may be represented by a simple alignment chart with straight parallel scales. We are at liberty to choose the spacing and graduation of two of these scales at pleasure; the position and graduation of the third scale is then fixed. The main consideration in the choice is to keep the chart reasonably compact and to have the working length of each scale approximately the same. In view of this, the following choice of scales proves satisfactory, each scale being specified in terms of the  $x$  and  $y$  coordinates of a point on the scale.

Scale	$x$	$y$
$\sigma$	+5	$(5/4)\sigma - 5$
$M$	+4	$\log M - 4$
$f/f_0$	0	$(15/2) \log f/f_0$

That this choice gives an alignment chart which satisfies the equation in question may be verified by evaluation of the determinant

$$\begin{vmatrix} 5 & (5/4)\sigma - 5 & 1 \\ 4 & \log M - 4 & 1 \\ 0 & (15/2) \log f/f_0 & 1 \end{vmatrix}$$

This provides for the first half of the double alignment chart, which lies to the right of the pivot scale in Fig. 1.

The same considerations lead to the following choice of scales for the alignment chart for Equation 26, which constitutes the other, left-hand, half of the nomogram.

Scale	$z$	$u$
$f/f_0$	0	$(15/2) \log f/f_0$
$\rho$	$-7/5$	$6 \log \varphi(\rho) + 3/5$
$h$	$-7$	$-10 \log (1 + h) + 3$

It should be noted that the  $\rho$  scale is double valued, one set of values corresponding to prolate ellipsoids ( $\rho < 1$ ) and the other to oblate ellipsoids ( $\rho > 1$ ).

We are not of course directly interested in the parameter  $\sigma$ , but only in the variables  $\bar{v}_{20}$  and  $s_{20}$  on which it depends. To any value of  $\sigma$  there corresponds an infinite number of pairs of values of these variables, and to any pair of these there corresponds a single value of  $\sigma$ . It is possible to introduce  $s_{20}$  and  $\bar{v}_{20}$  into the alignment chart by means of contours. For this purpose we require three families of contours, one for  $s_{20}$ , one for  $\bar{v}_{20}$ , and one for  $\sigma$ . Two of these may be chosen at will and the other then follows, although of course any choice must be such that the  $\sigma$  contours intersect the  $\sigma$  scale in accordance with the already fixed graduation of that scale. We may conveniently choose the  $\sigma$  contours as horizontal straight lines, perpendicular to the  $\sigma$  scale. Similarly we choose the  $\bar{v}_{20}$  contours as straight evenly spaced vertical lines parallel to the  $\sigma$  scale. The  $s_{20}$  contours are then fixed and have the appearance shown in Fig. 1. In the alignment chart it is unnecessary to draw in the  $\sigma$  contours. The appropriate point on the  $\sigma$  scale is obtained simply by projecting the point given by the intersection of the corresponding  $s_{20}$  and  $\bar{v}_{20}$  contours on the  $\sigma$  scale. If either  $s_{20}$  or  $\bar{v}_{20}$  is known, the other follows on the same basis when a point on the  $\sigma$  scale is fixed. It will be noticed that the contour for  $\bar{v}_{20} = 0.74$  coincides with the  $\sigma$  scale of the alignment chart.

It is a very simple thing, on the basis of Equation 25, to add to the right-hand half of the nomogram a scale in connection with contours which

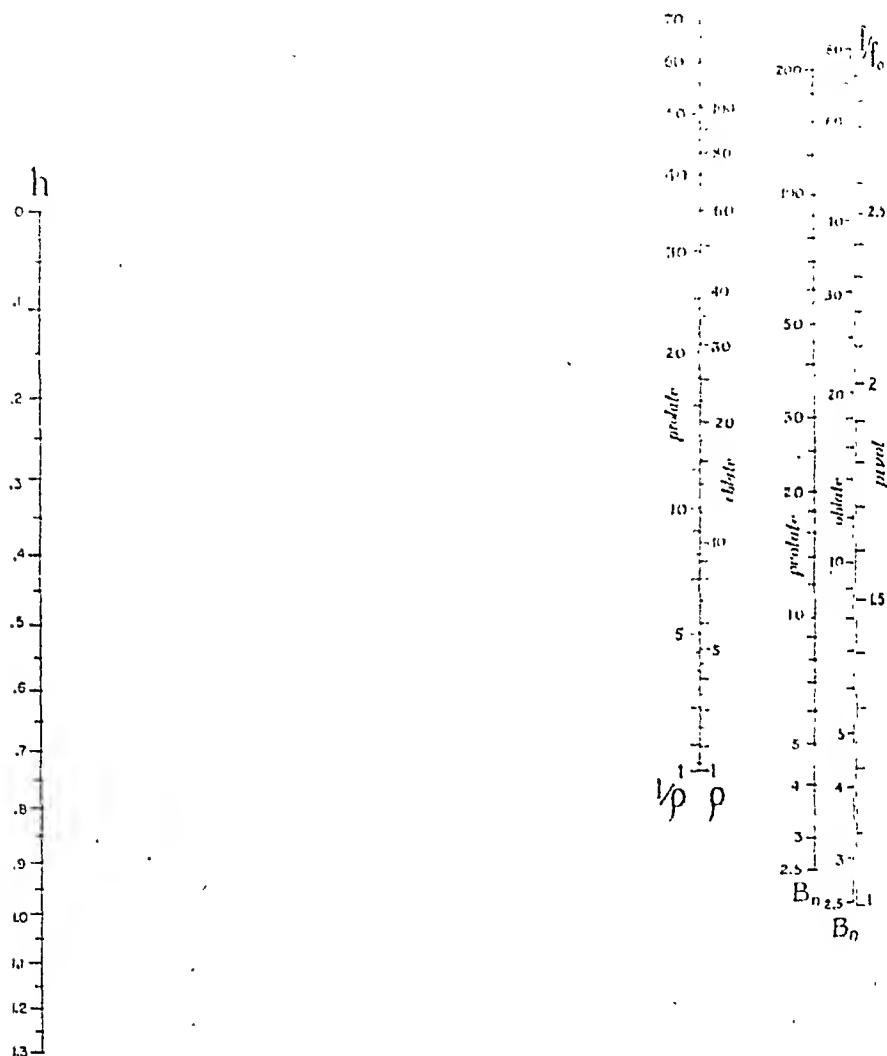


FIG. 1, *a*

FIG. 1, *a* AND *b*. Nomogram for sedimentation constant (*s*), diffusion constant (*D*), partial specific volume ( $\bar{v}$ ), molecular weight (*M*), frictional ratio (*f*/*f*<sub>0</sub>), viscosity increment (*B*<sub>η</sub>), axial ratio (*ρ*), and hydration (*h*). Values of those variables which depend on temperature are for 20°.

gives *D*<sub>20</sub>. The position and graduation of this scale are of course fixed by the previous choice of the scales for *M* and *f*/*f*<sub>0</sub>. For this purpose we treat

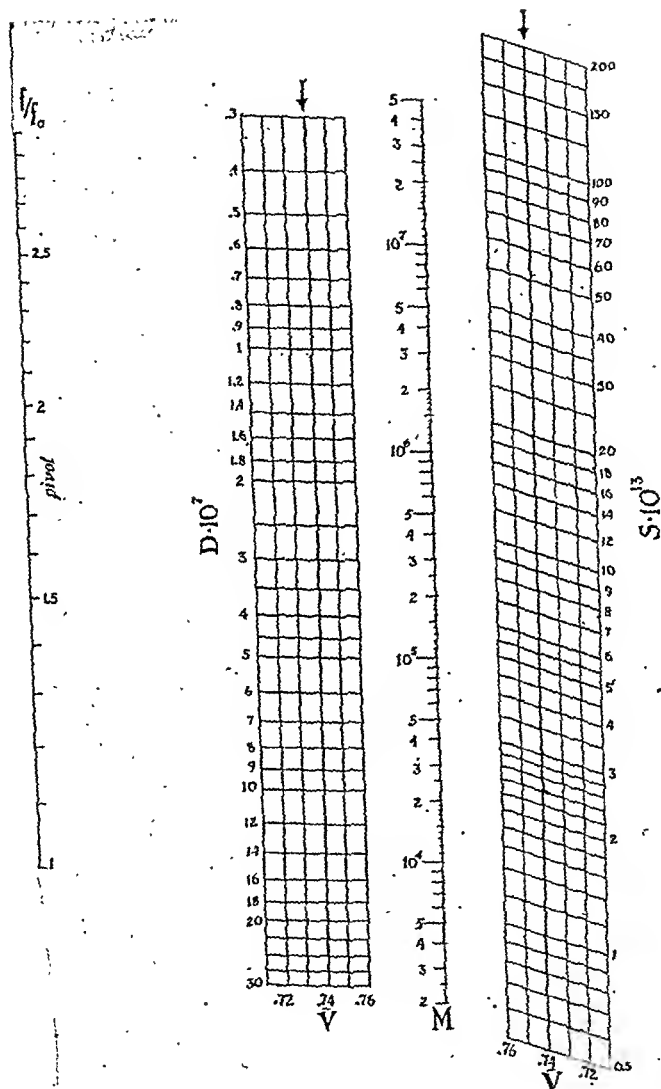


FIG. 1, b

the expression  $(13.6148 + 3 \log D_{20} + \log \bar{v}_{70})$  in Equation 25 just as we did the corresponding expression in parentheses in Equation 24. If we



denote this expression by  $\delta$ , the  $\delta$  scale is found to be given by the equations  $x = 20/7$ ,  $y = -(5/7)\delta - 20/7$ . The  $\delta$  and  $\bar{v}_{20}$  contours are chosen as horizontal and vertical lines respectively.

We now have to consider the possibility of adding a scale for the viscosity increment on the basis of Equation 27. Since Equation 27 involves only  $h$  and  $\rho$  in addition to  $B_\eta$ , this scale should belong to the left-hand half of the alignment chart. In general it would not be possible to add a scale for the viscosity increment, but, owing to a particularly simple relation between  $\psi(\rho)$  and  $\varphi(\rho)$ , the problem becomes solvable. It is found, namely, that although  $\varphi(\rho)$  and  $\psi(\rho)$  are both complicated functions of  $\rho$ , nevertheless  $\log \psi(\rho)$  is linear in  $\log \varphi(\rho)$  for both prolate and for oblate ellipsoids to a high degree of approximation, probably well in excess of that involved in treating the molecules as ellipsoids of revolution. For prolate ellipsoids the relation is expressed by  $\log \psi(\rho) = 0.398 + (3.92 \pm 0.01) \log \varphi(\rho)$  and for oblate ellipsoids by  $\log \psi(\rho) = 0.398 + (3.06 \pm 0.01) \log \varphi(\rho)$ . Values of  $\log \psi$  calculated from these equations agree with the values given by Simha to approximately 1 per cent or better in the case of oblate ellipsoids and 2 per cent or better in the case of prolate ellipsoids as  $\rho$  varies from 1 to 100 or  $1/100$ . If we make use of these equations in connection with Equation 27, we obtain, for prolate ellipsoids  $(\log B_\eta - 0.398) - \log(1 + h) - 3.92 \log \varphi(\rho) = 0$  and for oblate ellipsoids  $(\log B_\eta - 0.398) - \log(1 + h) - 3.06 \log \varphi(\rho) = 0$ . The choice of scales for  $h$  and  $\rho$  having been already made, two scales are fixed for  $B_\eta$  in accordance with these equations, one for prolate, the other for oblate, ellipsoids. The equations of these scales are as follows: prolate,  $x = -0.3879$ ,  $y = 1.8079 \log \eta - 0.5533$ ; oblate,  $x = -0.0343$ ,  $y = 2.442 \log \eta - 0.9572$ .

Thus we obtain the double alignment chart shown in Fig. 1. This embodies the relations contained in Equations 24, 25, 26, and 27, and consists of eight scales and associated contours. Of these the  $f/f_0$  scale is the common scale which serves as pivot and belongs to each of the two single alignment charts of which the nomogram is composed. The  $M$ ,  $s$ , and  $D$  scales (with associated contours) belong to one of these component charts; and  $h$ ,  $\rho$ , and two  $B_\eta$  scales to the other. It should be realized that it is only the *relative* spacing of the scales from the  $f/f_0$  scale in each single alignment chart which is of significance; thus since  $x = 0$  for the  $f/f_0$  scale, the values of  $x$  which define the positions of the other scales in each half of the nomogram are of relative significance only and may be multiplied by any desired factor irrespective of what is done for the scales of the other half of the nomogram. This self-evident proposition might be justified formally by invoking the properties of determinants.

Unfortunately no simplifying relationship between  $\chi_1(\rho)$ ,  $\chi_2(\rho)$ , and  $\varphi(\rho)$  makes it possible to include either  $\tau_1$  or  $\tau_2$  in the alignment chart we

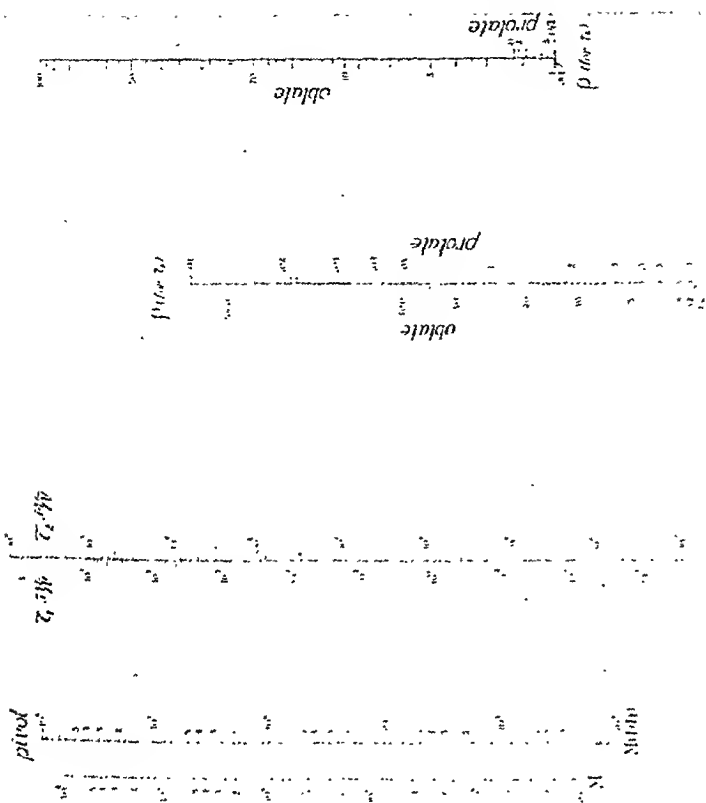


FIG. 2. Nomogram for relaxation times ( $\tau_1$  and  $\tau_2$ ), axial ratio ( $\rho$ ), molecular weight ( $M$ ), and hydration ( $h$ ). Values of  $\tau_1$  and  $\tau_2$  are for  $20^\circ$ .

have just been discussing. Indeed, from the point of view of constructing an alignment chart, the situation as regards Equations 28 and 29 is not very satisfactory. If we regard  $(\tau_1)_{20}/\bar{v}_{20}$ ,  $(\tau_2)_{20}/\bar{v}_{20}$ ,  $M(1 + h)$ , and  $\rho$

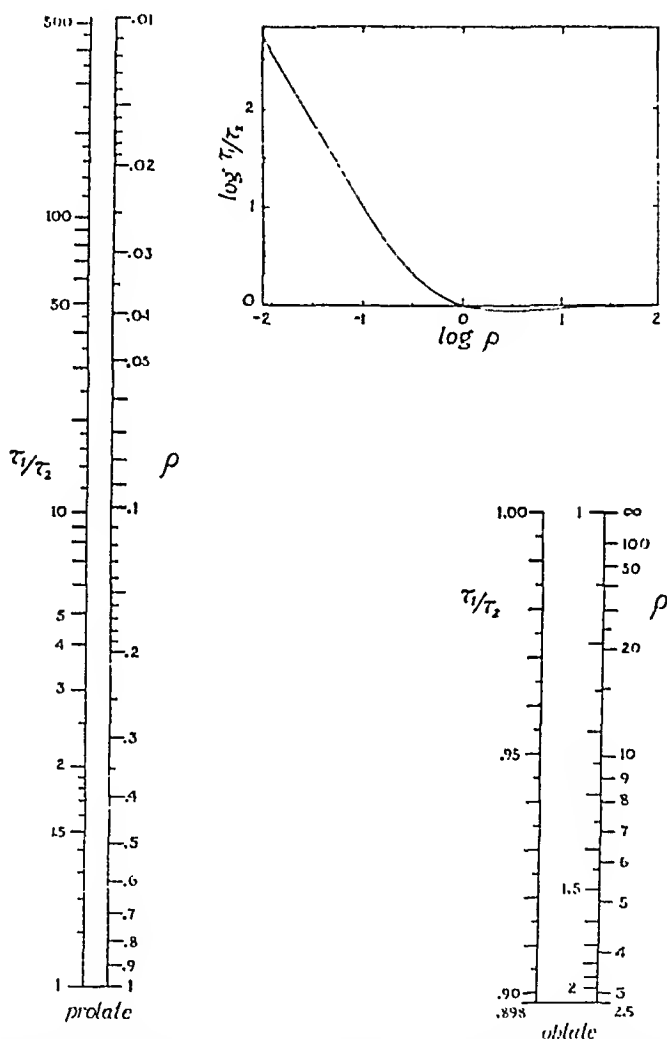


FIG. 3. Ratio of the two relaxation times ( $\tau_1/\tau_2$ ) as a function of axial ratio ( $\rho$ ) for oblate and prolate ellipsoids, calculated from the equations given by Perrin.

as the variables, we have two equations for the determination of four unknowns, only two of which can therefore be independent. If the nature of the relationships were suitable, it would be possible to construct a simple

single alignment chart with four scales. This would in fact be possible if  $\log \chi_1(\rho)$  were linear in  $\log \chi_2(\rho)$ . Such, however, is not the case, these two functions being extremely complicated, and indeed in certain ranges double valued. However, a fairly satisfactory representation is possible in terms of a double alignment chart. This is shown in Fig. 2 in which the  $M(1+h)$  scale is a common scale, which also serves as the pivot scale. One-half of Fig. 2 then corresponds to Equation 28 and contains scales for  $(\tau_1)_{20}$  and  $\rho$ ; and the other half corresponds to Equation 29 and has scales for  $(\tau_2)_{20}$  and  $\rho$ .<sup>2</sup> The unusual thing about the chart lies in the fact that there are two separate scales for  $\rho$ , one in each half. One of these is graduated on the basis of  $\chi_1(\rho)$ ; the other on the basis of  $\chi_2(\rho)$ . As in the case of all double alignment charts, the state of the system is represented by two straight lines which intersect on the pivot scale ( $M(1+h)$  scale).

TABLE I

*Definition of Scales of Alignment Chart for Relaxation Times and Related Properties*

Scale	$z$ (relative values)	$y$
$M$ .....	-1	$0.9 \log M - 2.88$
$h$ .....	-10	$-9 \log (1+h) + 2.7$
$M(1+h)$ .....	0	$\log M(1+h) - 3.5$
$\tau_1 \times 0.74/\bar{v}$ .....	+1	$0.6 \log \left( \tau_1 \frac{0.74}{\bar{v}} \right) + 4.6667$
$\tau_2 \times 0.74/\bar{v}$ .....	+1	$0.73 \log \left( \tau_2 \frac{0.74}{\bar{v}} \right) + 6.2317$
$\rho$ (for $\tau_1$ ).....	2.5	$1.5 \log \chi_1(\rho) - 1.1377$
" (" $\tau_2$ ).....	3.704	$2.704 \log \chi_2(\rho)$

Ordinarily this would mean that three variables must be specified to fix the state of the system. In the present case, however, owing to the fact that there is a scale for  $\rho$  in each half of the nomogram, only two variables need be specified: these may be any two of the four quantities  $M(1+h)$ ,  $(\tau_1)_{20}$ ,  $(\tau_2)_{20}$ , and  $\rho$ . If these happen to be  $(\tau_1)_{20}$  and  $(\tau_2)_{20}$ , it may require a number of trials to fix the position of two straight lines which intersect on the  $M(1+h)$  scale, cut the  $(\tau_1)_{20}$  and  $(\tau_2)_{20}$  scales correctly, and intersect the two  $\rho$  scales at corresponding points; *i.e.*, points giving the same value of  $\rho$ . This difficulty is overcome by reference to Fig. 3 which gives in the form of a graphical table values of  $\tau_1/\tau_2$  in relation to  $\rho$ .

It should be noticed that there are two general possibilities, one for the assumption that the molecules are prolate ellipsoids, in which case we must

<sup>2</sup> We speak of the two "halves" of the chart for convenience. Actually the scales of the two single alignment charts both lie on the same side of the pivot scale.

identify  $\tau_1$  with the longer relaxation time, the other for the assumption that the molecules are oblate ellipsoids, in which case we must identify  $\tau_1$  with the shorter relaxation time. However, the nature of the functions

TABLE II

*Perrin's Functions for Ratios of the Two Relaxation Times of an Ellipsoid of Revolution to the Relaxation Time of a Sphere of Equal Volume*

$\rho$  denotes axial ratio;  $\chi_1(\rho)$  applies to the relaxation time for a moment parallel to the axis of revolution;  $\chi_2(\rho)$  applies to the relaxation time for a moment perpendicular to the axis of revolution.

Oblate ellipsoids			Prolate ellipsoids		
$\rho$	$\chi_1(\rho)$	$\chi_2(\rho)$	$\rho$	$\chi_1(\rho)$	$\chi_2(\rho)$
1	1	1	1	1	1
1.1	0.9863	1.013	0.9	1.028	0.994
1.2	0.9831*	1.030	0.843	1.051	0.992*
1.3	0.9876	1.049	0.8	1.077	0.993
$\sqrt{2}$	1	1.077	$\sqrt{0.5}$	1.151	1
1.5	1.013	1.100	0.7	1.156	1.001
2	1.132	1.256	0.6	1.287	1.020
3	1.465	1.626	0.5	1.505	1.050
4	1.843	2.026	0.4	1.893	1.094
5	2.240	2.437	0.3	2.670	1.156
6	2.635	2.845	0.25	3.395	1.190
7	3.056	3.271	0.2	4.642	1.226
8	3.47	3.69	0.15	7.103	1.259
10	4.305	4.534	0.1	13.369	1.295
12	5.14	5.37	0.09	15.85	1.301
14	6.00	6.22	0.08	19.10	1.307
15	6.407	6.649	0.07	23.86	1.312
16	6.82	7.05	0.06	30.95	1.317
18	7.67	7.91	0.055	36.3	1.319
20	8.520	8.768	0.05	41.82	1.321
30	12.74	13.01	0.045	50.0	1.323
40	16.98	17.25	0.04	61.06	1.325
50	21.24	21.49	0.035	77.45	1.327
60	25.47	25.74	0.03	100.1	1.328
70	29.72	29.98	0.025	138.4	1.330
80	33.90	34.23	0.02	203.0	1.331
90	38.0	38.47	0.015	338.8	1.332
100	42.44	42.71	0.01	694.7	1.333

\* Minimum.

$\chi_1$  and  $\chi_2$  is such that for oblate ellipsoids  $\tau_1$  and  $\tau_2$  never differ by more than about 10 per cent, regardless of  $\rho$ . It is doubtful whether at the present time there are any experimental results good enough to warrant resolution in terms of two relaxation times so close together. If only

one relaxation time is observed (as for example in the case of hemoglobin) this in itself may mean either that the molecules are oblate and of almost any axial ratio or that they are prolate but nearly spherical or that they are of any axial ratio but with the electric moment parallel to one of the axes.

In making the actual choice of scales for this alignment chart we have made the  $\tau_1$  and  $\tau_2$  scales coincide, each being a single valued scale. In reality these scales are for  $(\tau_1)_{20} \times 0.74/\bar{v}_{20}$  and  $(\tau_2)_{20} \times 0.74/\bar{v}_{20}$ , 0.74 being close to the mean value for the partial specific volume for proteins.<sup>3</sup> Variations in partial specific volume for different proteins being relatively small, it seems unnecessary to introduce a set of contours as in the case of sedimentation and diffusion constants.

We have also introduced into the alignment chart two additional scales, one for  $h$  and one for  $M$  on the basis of the relation

$$\log M + \log (1 + h) = \log M(1 + h)$$

Actually this converts it into a triple alignment chart but introduces very little extra complexity and adds considerably to the convenience of the nomogram.

The definition of all the scales is given in Table I.

Table II gives numerical values of  $\chi_1$  and  $\chi_2$  in relation to  $\rho$ , calculated from the equations given by Perrin for both oblate and prolate ellipsoids. It is interesting to observe how the values of these functions pass through minima, less than unity, for axial ratios different from 1.

### Illustrations

In concluding we shall illustrate the use of these nomograms by one or two examples, beginning with myoglobin. Theorell, soon after he isolated this protein in 1932, undertook a study of its molecular weight in the centrifuge (9). Preparations of myoglobin from kidney of the horse and skeletal muscle of the cat were found to be monodisperse in the centrifuge at pH greater than 6 and had a value of  $s_{20} = 1.9$  to  $2.1 \times 10^{-13}$ . In contrast to this, certain preparations of myoglobin from horse hearts appeared to contain two components, at pH greater than 6, one with  $s_{20} \cong 2 \times 10^{-13}$ , and the other with  $s_{20} \cong 4 \times 10^{-13}$ , although other preparations from horse hearts were monodisperse with  $s_{20} = 2 \times 10^{-13}$ . Measurements of the diffusion constant were made on the more slowly centrifuging material.

<sup>3</sup> This really means that we rewrite Equation 28 in the form  $(\log(\tau_1)_{20} \frac{0.74}{\bar{v}_{20}} - \log 0.74 + 11.9059) - \log M - \log (1 + h)$  and treat  $(\tau_1)_{20} \frac{0.74}{\bar{v}_{20}}$  as a variable in place of  $(\tau_1)_{20}$ . The same is true of  $(\tau_2)_{20}$ .

The values obtained for  $D_{20}$  varied between 5.2 and  $11.7 \times 10^{-7}$ , corresponding to a range of molecular weight from 16,000 to 34,000. Equilibrium measurements in the centrifuge gave a molecular weight of about 35,000 for myoglobin prepared from horse kidney, but equilibrium measurements on myoglobin from horse hearts indicated that the material was polydisperse with a molecular weight ranging from 36,700 to 58,740. Further equilibrium measurements on other preparations of horse myoglobin gave values of the molecular weight from 8500 to 17,000, but these very low values were attributed to breakdown of the molecules as a result of bacterial action. Still other equilibrium measurements in which toluene was added gave  $M = 33,000$ . Some time later (1937) Polson ((8) p. 376) renewed the study of horse myoglobin and found  $s_{20} = 2.04 \times 10^{-13}$ , in agreement with many of the earlier results, but obtained  $D_{20} = 11.3 \times 10^{-7}$ . This would mean a molecular weight of 16,900. Polson also made equilibrium measurements on the same material and found the molecular weight to be 17,500. Still more recent studies have added to the data on myoglobin. In 1940, Roche and Vicil (7) carried out osmotic pressure determinations of myoglobin from the skeletal muscle of the horse and found  $M = 16,850$ . In 1942, Marey and Wyman (2) studied the dielectric properties of metmyoglobin from horse hearts. Their dispersion curves indicate a single relaxation time corresponding to  $\tau_{20} = 3.1 \times 10^{-8}$ . In the course of this work Marey and Wyman also had occasion to measure the viscosity of the protein in aqueous solution at 25° and a concentration of 2.32 gm. per 100 cc. The results, obtained with an Ostwald viscosimeter, give  $\eta/\eta_0 = 1.0759$ . If we use Theorell's value of 0.741 for the partial specific volume of the protein, this gives a viscosity increment of 4.42. Actually the value to be used in the nomograms is the limiting value of the viscosity increment at zero concentration. By analogy with other proteins we may expect this to be lower than that obtained at any finite concentration, and the effect in the present case should amount to perhaps 5 per cent or more. If we assume that the viscosity obeys the empirical Arrhenius equation  $\eta/\eta_0 = a^{bc}$ , where  $a$  and  $b$  are constants and  $c$  is the concentration of the protein in gm. per cc., it follows that  $(d \ln \eta/\eta_0)/dc = b \ln a = a$  constant, independent of concentration.  $b \ln a$ , however, is nothing but the limiting value approached by the indeterminate form  $(\eta/\eta_0 - 1)/c$  as  $c$  goes to zero. On this basis the limiting value of the viscosity increment may be estimated as  $2.303 \log 1.0759/(2.32 \times 0.741) = 4.25$ . The correct value of  $B_\eta$  therefore should be close to 4.2.

The observed values of the various quantities may be entered on the two nomograms. The results are shown in Fig. 4, in which each point or range of values is accompanied by a letter corresponding to the observer.

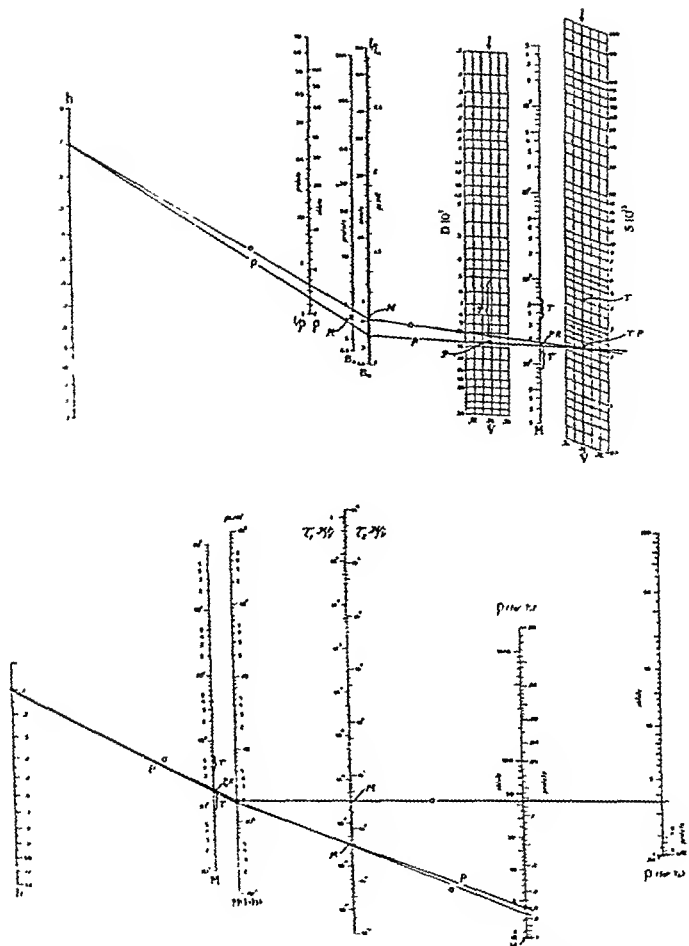


FIG. 4. Representation of the data on myoglobin. Observations of various investigators are indicated by initials as follows: *T*, Theorell; *P*, Polson; *R*, Roche and Vieil; *M*, Marey and Wyman. The straight lines represent the state of the molecule, in regard to all the data. Lines marked *p* correspond to the hypothesis of prolate ellipsoids; those marked *o* to that of oblate ellipsoids.

It is of course necessary to enter the viscosity increment on each of the two scales, one for oblate, the other for prolate, ellipsoids, since we have no way of knowing the shape of the myoglobin molecules in advance, although



the absence of two distinct relaxation times suggests that they are not highly elongated. We also enter the relaxation time twice, once as  $\tau_1$  once as  $\tau_2$ , although it is possible that the electric moment coincides with one of the two principal axes of the molecule, in which case the corresponding  $\tau$  drops out of the picture. Unfortunately nothing is known directly about the hydration of myoglobin, although by analogy with hemoglobin it might be expected to be about 20 per cent by weight, corresponding to  $h \cong 0.27$ .

It will be seen from Fig. 4 that a fairly consistent picture is obtained by regarding the myoglobin molecule as a prolate ellipsoid of revolution with an axial ratio of  $1/2.5 = 0.40$  and with its electric moment approximately parallel to the long axis, so that  $\tau_2$  drops out. The adjusted values of the various quantities are then as follows:  $s_{20} = 2.0 \times 10^{-13}$ ,  $D_{20} = 11.5 \times 10^{-7}$ ,  $M = 16,500$ ,  $B_\eta = 3.7$ ,  $(\tau_1)_{20} = 3.1 \times 10^{-8}$ ,  $\rho = 1/2.5 = 0.40$ ,  $h = 0.1$ ,  $f/f_0 = 1.11$ . It would be impossible to reconcile the results with the assumption of an electric moment parallel to the short axis. On the other hand an almost equally satisfactory interpretation would be to regard the molecule as a flattened ellipsoid with an axial ratio of 3.6. The adjusted values of the various quantities are then as follows:  $s_{20} = 2.0 \times 10^{-13}$ ,  $D_{20} = 10.3 \times 10^{-7}$ ,  $M = 18,000$ ,  $B_\eta = 4.1$ ,  $(\tau_1)_{20} = 3.0 \times 10^{-8}$ ,  $(\tau_2)_{20} = 3.3 \times 10^{-8}$ ,  $\rho = 3.6$ ,  $h = 0.1$ ,  $f/f_0 = 1.8$ . Between these alternatives there is really no strong basis for decision. One thing, however, is clear. The viscosity data absolutely rule out the possibility of a sedimentation constant of  $2 \times 10^{-13}$  and a molecular weight much above 19,000.

The significance of viscosity measurements is brought out by this nomographic treatment. Since the scales for  $B_\eta$  and  $f/f_0$  nearly coincide in the case of oblate ellipsoids, a knowledge of the viscosity increment is practically equivalent to a knowledge of  $f/f_0$ . Thus, for oblate ellipsoids, the viscosity increment serves as well as the diffusion constant to fix the molecular weight in connection with the sedimentation constant. In the case of prolate ellipsoids, an exact knowledge of the viscosity increment and a rough estimate of  $h$  serve to fix  $f/f_0$ . For example, if  $B_\eta$  is 4,  $f/f_0$  only varies from approximately 1.65 to 1.7 when  $h$  varies from 0.1 to 0.3. It thus appears that viscosity is a more significant quantity in connection with determinations of molecular weight than has often been realized. It is in many ways more amenable to exact measurements than the diffusion constant.

Our first example of the use of the nomograms has been one of the more confused cases. As a second example let us take hemoglobin, which is one of the best characterized of all the proteins. Its molecular weight, based on analytical data, is 66,700; that based on osmotic pressure is 67,000;

that obtained from equilibrium studies in the centrifuge is 68,000. It shows a single relaxation time,  $\tau_{20} = 9.6 \times 10^{-8}$ . Values of the other quantities are as follows:  $s_{20} = 4.41 \times 10^{-13}$  (4),  $D_{20} = 6.9 \times 10^{-7}$  (4),  $\bar{v}_{20} = 0.749$  (4),  $B_\eta = 5.1$  (4),  $h = 0.4$  (1). With the aid of the two alignment charts, the situation may be interpreted on the basis of either of the following compromises.

*Prolate Molecules, Moment Perpendicular to Long Axis*— $s_{20} = 4.41 \times 10^{-13}$ ,  $D_{20} = 6.5 \times 10^{-7}$ ,  $M = 65,000$ ,  $B_\eta = 4.9$ ,  $(\tau_1)_{20} = 9.5 \times 10^{-8}$ ,  $\rho = 1/2.7 = 0.37$ ,  $h = 0.4$ .

*Oblate Molecules*— $s_{20} = 4.41 \times 10^{-13}$ ,  $D_{20} = 6.5 \times 10^{-7}$ ,  $M = 65,000$ ,  $B_\eta = 4.6$ ,  $(\tau_1)_{20} = 11 \times 10^{-8}$ ,  $(\tau_2)_{20} = 13 \times 10^{-8}$ ,  $\rho = 2.7$ ,  $h = 0.4$ .

If we adopted the oblate interpretation, we might assume that the component of the moment corresponding to  $\tau_2$  is negligible.

As a final example we take the case of edestin, a protein somewhat less well characterized than either of the others we have been considering. Direct measurements of the molecular weight of this protein are not available. According to Oncley (4), the dielectric constant data are resolvable in terms of two relaxation times,  $\tau_{20} = 286 \times 10^{-8}$ ,  $\tau_{20} = 32 \times 10^{-8}$ . Other data given by Oncley are as follows:  $s_{20} = 12.8$  (or 14.6, from preliminary studies of Oncley),  $D_{20} = 3.93 \times 10^{-7}$ ,  $\bar{v} = 0.744$ . The two relaxation times are so widely different as to rule out the possibility of flattened molecules. We may therefore identify  $286 \times 10^{-8}$  with  $\tau_1$  and  $32 \times 10^{-8}$  with  $\tau_2$ . This gives  $\tau_1/\tau_2 = 8.9$ . From Fig. 4 we see that this corresponds to  $\rho = 0.112 = 1/8.9$ . Turning to Fig. 3, we then obtain at once  $M(1+h) = 270,000$ . In contrast to this the lower value of the sedimentation constant gives a molecular weight of 310,000 and  $f/f_0 = 1.21$ . If we assume no water of hydration ( $h = 0$ ), this may be seen from Fig. 2 to give  $\rho = 1/4.5 = 0.222$ . Thus there is a real discrepancy. A possible, though not very satisfactory, compromise would be to assume  $s_{20} = 12.8 \times 10^{-13}$ ,  $D_{20} = 3.4 \times 10^{-7}$ ,  $M = 360,000$ ,  $(\tau_1)_{20} = 210 \times 10^{-8}$ ,  $(\tau_2)_{20} = 41 \times 10^{-8}$ ,  $\rho = 1/6.25 = 0.16$ ,  $h = 0$ ,  $f/f_0 = 1.33$ . It is unfortunate that no viscosity data are available. As we have pointed out, a knowledge of viscosity may be decisive in settling just such issues as this.

These three examples show the convenience and value of the alignment charts given in Figs. 2 to 4 in interpreting and reconciling various data relating to the molecular weight of proteins, as well as in presenting in comprehensible form the somewhat complicated interrelations involving the various properties.

*Note*—It is impractical to reproduce in the *Journal* the nomograms at workable size. A limited number of planograph reproductions are available and copies may be obtained from the authors.

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# THE QUANTITATIVE RELATIONSHIP BETWEEN $\beta$ -HYDROXY-BUTYRIC ACID AND ACETOACETIC ACID IN BLOOD AND URINE\*

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Although the quantitative relationship between  $\beta$ -hydroxybutyric acid and acetoacetic acid in blood and urine has been the subject of extensive studies during the last four decades, we found no information as to the distribution of the two acids between corpuscles and plasma. To obtain this information for our current studies was the primary purpose of the work reported in this article.

It has been well recognized that the quantitative relationship between the two acids varies with changing conditions, but the factors which can cause such changes were scarcely studied systematically. In the present work we determined the ratio of  $\beta$ -hydroxybutyric acid to acetoacetic acid in the blood (both plasma and corpuscles) and urine of healthy and diabetic subjects in the postabsorptive state, precluding effects which absorption of food or the administration of insulin might exert.

## Blood

Since the concentration of ketone bodies in the blood of healthy individuals is very low (a maximum of 1 mg. per cent of total ketone bodies expressed as  $\beta$ -hydroxybutyric acid), 70 to 80 cc. of blood were required for the analysis of both whole blood and plasma.

For analysis 25 cc. of blood were deproteinized by dilution with 50 cc. of 0.3 N barium hydroxide and subsequent addition of 50 cc. of a 5 per cent solution of zinc sulfate. The remaining blood was centrifuged for 20 minutes and the cell volume was determined. Of the plasma 25 cc. were deproteinized in the same manner as the whole blood. In order to enhance the amount of protein-free filtrate, the precipitate was separated by centrifugation. Glucose was eliminated from the filtrate by Salkowski's method, solid anhydrous copper sulfate and calcium hydroxide being used in order to avoid further dilution. Equivalents of 10 to 12 cc. of blood or plasma (50 to 60 cc. of filtrate) were used for the determination of the  $\beta$ -hydroxybutyric and acetoacetic acids by a method previously described (1). In the cases of fasting and of diabetic subjects much smaller amounts of blood sufficed for these determinations. The concentrations of the

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ketone bodies in the corpuscles were calculated from the analytical data of whole blood and of plasma with knowledge of the cell volume.

Table I contains results obtained on the blood samples drawn from healthy young individuals. Samples 1 to 5 were taken directly before breakfast, 12 to 14 hours after the last meal; Samples 6 to 8 after fasting for various periods of time. Samples 4 and 5 were pooled specimens, each obtained from three subjects, while all the others were individual specimens.

The concentrations of the two acids (expressed in terms of  $\beta$ -hydroxybutyric acid), as given in Table I, were determined separately in whole blood and plasma. The amount of the total ketone bodies represents the sum of the two constituents, not the result of direct analysis. The term  $\beta$  ratio,

TABLE I  
*Ketone Bodies in Whole Blood and Plasma of Healthy Subjects in Postabsorptive State and after Fasting*

Sample No.	Condition	Plasma				Whole blood			
		Aceto-acetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio	Aceto-acetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio
		mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
1	Postabsorptive	0.43	0.93	1.36	68	0.40	0.60	1.00	63
2	"	0.23	0.65	0.78	83	0.23	0.39	0.62	63
3	"	0.04	0.11	0.15	73	0.09	0.14	0.23	61
4	"	0.25	0.70	0.95	74	0.16	0.36	0.52	69
5	"	0.11	0.27	0.38	71	0.08	0.19	0.27	70
6	Fasted 64 hrs.	21.8	66.1	87.9	75	24.0	44.4	68.4	65
7	" 62 "	4.86	22.9	27.7	83	5.36	18.0	23.4	76
8	" 38 "	5.51	12.9	18.4	70	4.85	8.25	13.1	63

suggested by Kennaway (2) to denote the value  $100 \times (\beta\text{-hydroxybutyric acid})/(\text{total ketone bodies})$ , was adopted for its convenient brevity.

The values in Table I convey three clear cut facts. First, it may be seen that the  $\beta$  ratio in whole blood varies between 60 and 75, a range that was generally observed by numerous workers. After prolonged fasting the  $\beta$  ratio is much the same as in the postabsorptive state. The second fact revealed in Table I is that the  $\beta$  ratio is appreciably higher in plasma than in whole blood, obviously due to the fact that the amount of acetoacetic acid in relation to the  $\beta$ -hydroxybutyric acid is considerably greater in the corpuscles than in the plasma. Thirdly, the concentration of total ketone bodies is appreciably higher in the plasma than in the whole blood, owing to an uneven distribution between corpuscles and plasma.

This uneven distribution becomes more obvious if one compares the

calculated values for corpuscles, presented in Table II, with the analytical data of plasma (Table I). It may be seen that, with the exception of one case (Sample 3), the concentration of total ketone bodies in the corpuscles is by and large only one-half as high as in the plasma. This statement cannot be based on the postabsorptive samples (Nos. 2 to 5), in which the total ketone body content of the blood is less than 1 mg. per cent; these results are inconclusive because the values calculated for the corpuscles from two determined quantities may be greatly distorted by analytical errors, especially if these should be additive. In Samples 6, 7, and 8, however, from subjects in which ketonemia was rather high, analytical errors could not essentially affect the data calculated for the corpuscles. As shown in Table II, in these samples the concentration of total ketone

TABLE II  
*Ketone Bodies in Blood Corpuscles Calculated from Data in Table I*

Sample No.	Cell volume	Acetoacetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$100 \times \frac{\text{ketones of cells}}{\text{ketones of plasma}}$	$\beta$ ratio
	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>		
1	50.0	0.46	0.32	0.78	57.4	41
2	46.5	0.34	0.11	0.45	57.7	25
3	46.6	0.15	0.17	0.32	213.3	53
4	42.0	0	0	0	0	0
5	41.2	0.02	0.07	0.12	0.1	58
6	50.0	26.1	22.7	48.8	54.9	42
7	43.5	6.00	11.7	17.7	64.0	66
8	44.3	4.01	2.41	6.42	34.8	38

bodies in the corpuscles was, respectively, but 54.9, 64.0, and 34.8 per cent as high as in the plasma.

This distribution ratio between corpuscles and plasma, however, applies only to the total ketone bodies. When acetoacetic acid and  $\beta$ -hydroxy-butyric acid are regarded separately, it may be noted that the relationship between the two acids is substantially different in corpuscles on the one hand and plasma on the other. Namely, while in the plasma acetoacetic acid represents only about 20 per cent of the total ketone bodies, in the corpuscles it increases to 50 per cent and higher. Thus it happens that whereas the concentration of total ketone bodies may be only half as high in corpuscles as in plasma, acetoacetic acid concentration in the corpuscles may equal and even exceed that in the plasma.

Diabetic patients with varying degrees of ketosis exhibit in general a picture much like our healthy subjects. In Table III are presented the results on eight patients. Samples 2, 3, and 5 were obtained from newly

diagnosed patients who had never received treatment with insulin; Samples 1, 6, 7, and 8 were obtained when the patients were in a state of coma. To none of them was insulin administered for at least 24 hours prior to our observations, and all were in the postabsorptive state.

As may be seen in Table III (Samples 1, 2, 4, and 5), the plasma showed a higher concentration of ketone bodies than whole blood, just as in healthy individuals. In Samples 6, 7, and 8, however, the distribution was inverted; these were incidentally from patients in coma who died. As to the  $\beta$  ratio, this, as in our healthy subjects, was higher in the plasma than in whole blood, showing the relative preponderance of acetoacetic acid in the corpuscles. Yet variations were considerable. In Sample 2 (Table III),

TABLE III  
*Ketone Bodies in Whole Blood and Plasma of Diabetic Patients in Postabsorptive State*

Sample No.	Plasma				Whole blood			
	Aceto-acetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio	Aceto-acetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio
	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
1*	95.5	253.0	348.5	73	98.5	198.0	296.0	67
2	5.92	21.0	26.9	78	7.30	16.4	23.7	69
3	1.31	3.47	4.78	73	1.31	3.47	4.78	73
4	10.0	59.8	78.8	76	24.8	43.2	68.0	68
5	7.20	31.5	38.7	81	7.70	17.7	25.4	70
6*	34.7	154.0	188.7	82	63.2	132.0	195.2	68
7*	59.4	82.9	142.3	57	77.0	83.6	160.6	52
8*	35.7	121.0	156.7	78	39.8	124.0	163.8	76

\* Specimens obtained when patients were admitted to various hospitals in diabetic coma.

for instance, the corpuscles contained 9.5 mg. per cent of acetoacetic acid and the  $\beta$  ratio was 50, whereas the plasma contained only 5.9 mg. per cent and showed a  $\beta$  ratio of 78 (total ketone bodies in the corpuscles were 19 mg. per cent, in the plasma 26.9 mg. per cent). This is in line with our findings in healthy fasting subjects. In Sample 5 (obtained from an untreated diabetic) an extreme picture presented itself, in that the corpuscles contained 29.1 mg. per cent of acetoacetic acid and no  $\beta$ -hydroxybutyric acid at all.

### Urine

In order to obtain a base-line for our studies, we analyzed a number of urines of healthy and diabetic subjects, always ascertaining that the samples were excreted in the postabsorptive state and with the exclusion of any

insulin effect. All subjects voided upon rising in the morning, discarded the urine, then collected the urine that was excreted from that time until breakfast. The diabetic subjects have had no insulin injections for at least 24 hours preceding the experiments.

For analysis the urines were rendered free of sugar by copper sulfate-lime treatment, according either to Salkowski's or Van Slyke's technique. For further purification the sugar-free filtrates were treated with basic lead acetate and disodium phosphate, the same reagents that we employed for the desaccharification of blood.

TABLE IV  
*Relationship between Acetoacetic and  $\beta$ -Hydroxybutyric Acids in Urine in Postabsorptive State*

Sample No.	Condition	Acetoacetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio
		mg. per cent	mg. per cent	mg. per cent	
1	Postabsorptive	0.21	1.47	1.68	87
2	"	0.34	1.07	1.41	76
3	"	0.23	1.47	1.70	87
4	"	0.43	1.78	2.11	80
5	"	0.31	0.60	0.91	67
6	"	0.27	1.43	1.70	84
7	Fasted 62 hrs.	96.5	131.0	227.5	58
8	" 38 "	30.6	74.7	105.3	72
9	" 64 "	70.1	150.0	220.1	68
10	Diabetic, post-absorptive	48.5	167.0	215.5	76
11	" "	54.7	120.0	174.7	69
12	" "	14.1	41.4	55.5	75
13	" "	25.0	57.0	82.0	70
14	" "	82.9	144.0	226.9	63
15	" "	80.4	265.0	345.4	77
16	" "	189.0	502.0	791.0	76

After this double precipitation urines still contain substances that interfere with the analysis of the ketone bodies. When the method of Van Slyke and Fitz (3) is used, this interference is negligible in cases with substantial degrees of ketonuria. In the instance of normally fed healthy subjects, however, who excrete only 20 to 40 mg. of ketone bodies (in terms of  $\beta$ -hydroxybutyric acid) per 24 hours, the selectiveness of the Denigès reagent proves to be insufficient and the analytical results are misleading. In particular the  $\beta$ -hydroxybutyric acid values are too high, being multiples of the values obtained with the triple oxidation and distillation method of Shaffer and Marriott (4). We used, therefore, the method of Shaffer and



Marriott adapted to a micro scale. Subsequently we found a somewhat shorter procedure. Namely, we used our micromethod as devised for blood analysis, but complemented it with an added step which consists in dissolving the Denigès precipitate and distilling off the acetone after the solution is rendered alkaline. The results obtained by this technique showed close agreement with those of the Shaffer-Marriott procedure.

In Table IV are presented our findings on three groups of subjects. Samples 1 to 6 were obtained from healthy subjects in the postabsorptive state. In view of the low ketone body concentrations in these urines, the purified portions used for an analysis represented not less than 15 cc. of urine, so that the amounts of acetone actually measured were sufficient to secure a fair degree of analytical accuracy. As may be noted, the normal urines in the postabsorptive state contained acetoacetic and  $\beta$ -hydroxybutyric acids in a rather uniform relationship; the lowest  $\beta$  ratio was 67, the highest 87, the average 80. Samples 7 to 9 were from healthy young persons who had fasted for at least 36 hours. The  $\beta$  ratio here was of the same general order as in the postabsorptive urines of normally fed subjects. Samples 10 to 16 were obtained from seven diabetic patients. The  $\beta$  ratio in these samples varied between 63 and 77, with an average of 72. These values are somewhat lower than we found in normal subjects, but in lack of statistical material, the differences can scarcely be considered as significant.

This range of variations in the  $\beta$  ratio is notably narrower than most workers in the past have described. This we ascribe to the fact that the urine samples in our work were collected under standardized conditions, while earlier investigators, with a few exceptions, paid no attention to the possible influence of food intake and of insulin injections. As we shall show in subsequent reports, both of these factors profoundly affect the  $\beta$  ratio.

#### SUMMARY

Studies concerning the distribution and quantitative relationship of acetoacetic and  $\beta$ -hydroxybutyric acids in normal human blood and urine in the postabsorptive state gave the following results.

1. The concentration of ketone bodies is about twice as high in the plasma as in the corpuscles of the blood.

2. While in plasma  $\beta$ -hydroxybutyric acid predominates, so that acetoacetic acid amounts to only one-fifth of the total ketone bodies, in corpuscles acetoacetic acid constitutes one-half and often a greater portion of the ketone bodies; there are cases in which corpuscles contain acetoacetic acid but no  $\beta$ -hydroxybutyric acid at all.

3. The urine of normally fed healthy individuals always contains small

amounts of both acetoacetic and  $\beta$ -hydroxybutyric acid; the  $\beta$  ratio  $100 \times (\beta\text{-hydroxybutyric acid})/(\text{total ketone bodies})$  varies within a rather narrow range.

In diabetic patients the relationships are the same as in healthy persons. This applies to blood as well as to urine, provided that the samples are obtained in the postabsorptive state and with the preclusion of insulin action.

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## METABOLISM OF A PARAFFIN\*

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The studies of Channon and his collaborators (1, 2) on the absorption of paraffin hydrocarbons from the gastrointestinal tract led to the startling conclusion that *n*-hexadecane was not only absorbed but was catabolized to give undetermined products. Despite an early observation to the contrary by Bradley and Gasser (3), the prevalent belief prior to the work of Channon was that the aliphatic saturated hydrocarbons were completely unabsorbed and metabolically completely inert. By comparing the amount of unsaponifiable material excreted with the amount of hexadecane fed, El Mahdi and Channon (1) demonstrated that this hydrocarbon was absorbed by rats to the extent of 50 to 100 mg. per day. They were further able to show an increase in the amount of unsaponifiable material and a decrease in the iodine number of this fraction in various tissue lipids after the administration of hexadecane. They took this as evidence for the deposition of the hydrocarbon in the body. Later, in experiments on cats, Channon and Devine (2) succeeded in isolating hexadecane from tissue lipids after a prolonged feeding of this material. They noted that the total recovery of hydrocarbon from feces and tissues was much less than the amount fed and concluded that hexadecane was converted to other unidentified products in the animal body. The site of this conversion they assumed to be the liver, basing this assumption on the observation that there was little or no increase in the quantity of unsaponifiable material in that organ after the ingestion of hexadecane.

The purpose of the present study was to determine whether normal aliphatic hydrocarbons may be oxidized, in the animal body, to fatty acids. In line with previous investigations from this laboratory (4, 5), in which the absorption and metabolism of the 16-carbon fatty acid and the analogous alcohol had been studied with the aid of isotopic hydrogen, it was determined to prepare and feed deuterio hexadecane under the same general conditions as in the experiments with deuterio palmitic acid and deuterio cetyl alcohol. It had been shown in these earlier experiments (5) that cetyl alcohol was efficiently absorbed and converted to palmitic acid by the rat, and it was hoped that, if a similar conversion occurred in the case of hexadecane, it should be demonstrable.

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Deuterio hexadecane was prepared for this purpose by the Kolbe reaction, electrolysis of potassium  $\alpha,\beta$ -didenterio pelargonate. The absence of exchange reaction between the hydrogen in the solvent and that on the  $\alpha$ -carbon of the acid under the conditions of the Kolbe synthesis has already been demonstrated for the synthesis of ethane from acetate (6, 7) and was found also to be true in the present synthesis. The product, therefore, must have been 7,8,9,10-tetradeuterio hexadecane.

An otherwise well rounded diet, the same as had been used in previous experiments (4), was mixed with 0.75 per cent of deuterio hexadecane and fed *ad libitum* to two growing rats until 200 gm. had been consumed, which required 9 days. The rats were then killed, gastrointestinal tracts removed and combined with the accumulated feces, and the livers removed for separate study. After alkaline hydrolysis of each portion, saponifiable and unsaponifiable fractions were isolated. The fatty acids were further separated over the lead salts into solid and liquid fractions. The unsaponifiable portions were separated into alcoholic and non-alcoholic fractions over the sodium salts of the hemiesters of succinic acid. The weights and corrected deuterium contents of each fraction are given in Table I.

From these data it is apparent that hexadecane is well absorbed from the gastrointestinal tracts of rats. The deuterium analysis of the non-alcoholic fraction of the fecal unsaponifiable matter indicates that, of the 1500 mg. fed, only  $132 \times 0.437 = 58$  mg. were unabsorbed. This means that about 80 mg. of hexadecane were absorbed per rat per day, a finding in agreement with that of El Mahdi and Channon (1). That this absorption took place prior to bacterial alteration of the molecule in the intestine is indicated by the high isotope content of the analogous fraction from the bodies of the rats. This fraction, however, though rich in isotope, is small in amount, accounting for only 38 mg. of the material fed. Precisely as noted by Channon, the hydrocarbon must have been catabolized in the body.

The finding of a significant though low concentration of  $D_2O$  in the body water, as has been found when deuterio fatty acids were fed (4), is in agreement with the idea of catabolic degradation of the hydrocarbon; the finding of a notably higher isotope concentration in the fatty acids of the carcass<sup>1</sup> at once indicates the catabolic route. About 15 per cent of the isotope fed as hexadecane was recovered in the fatty acids of the carcass. To explain this it is necessary to assume an oxidative process, presumably attacking a terminal methyl group, in which hexadecane is converted into palmitic acid. The oxidation of a carbon-bound methyl group to a carboxyl group has already been postulated in the theory of  $\omega$  oxidation. The

<sup>1</sup> For want of a better term, the word "carcass" is herein employed to mean the body of the animal less the liver and gastrointestinal tract.

present instance is perhaps more surprising in view of the notorious stability of saturated aliphatic hydrocarbons toward oxidizing agents *in vitro*.

It is not possible, from the present experiment, to state conclusively the site of this oxidation. That it did not occur exclusively in the gastrointestinal tract is shown by the fact that the isotope content of the fatty acids is much higher in the liver than in the feces. The deuterium concentration in the fecal fatty acids was slightly lower than that in the carcass

TABLE I

*Weight and Isotopic Composition of Fractions Isolated*

Two growing male rats were fed a total of 1.5 gm. of deuterio hexadecane, containing 9.7 atom per cent excess deuterium, over a period of 9 days. The isotopic composition is given (A) as the actual analytical figure and (B) recalculated on the basis of 100 atom per cent in the hexadecane fed.

Source of fraction	Fraction isolated	Weight	Deuterium content	
			A	B
		gm.	atom per cent	atom per cent
Feces + intestinal tracts	Fatty acids	2.161	0.11	1.1
	Solid		0.15	1.5
	Liquid		0.08	0.8
	Unsaponifiable	0.667		
	Non-alcoholic	0.132	4.24	43.7
	Alcoholic	0.417	0.06	0.6
Livers	Fatty acids	0.233	0.41	4.2
	Unsaponifiable	0.016		
Carcasses*	Fatty acids	15.272	0.15	1.5
	Solid		0.20	2.1
	Liquid		0.09	0.9
	Unsaponifiable	0.505		
" + livers	"	0.521		
	Non-alcoholic	0.256	1.44	14.9
	Alcoholic	0.254	0.06	0.6
Body water			0.04	0.4

\* Body less the liver and gastrointestinal tract.

fatty acids and probably resulted from normal fatty acid excretion. These findings, together with the observation that no large amount of unoxidized hydrocarbon accumulated in the liver (as also noted by Channon and Devine (2)), indicate the likelihood of the liver being the site of the oxidation under consideration.

No particular significance should be attached to the deuterium concentrations noted in the two alcoholic fractions isolated. These may well be due to the contamination of these crude mixtures by traces of isotopic hexadecane.

It is of interest to compare the fate of the three 16-carbon compounds, hexadecane, cetyl alcohol, and palmitic acid. All three of these compounds labeled with deuterium have now been fed to growing rats on the same basal diet, and for about the same length of time (4, 5). At least when fed in small doses, all three are readily absorbed. All three give rise to  $D_2O$  in the body water. In each case, the liver fatty acids are rich in deuterium, and the deuterium concentration in the carcass fatty acids is between one-half and one-third that in the liver fatty acids.

These results, obtained when small amounts of hydrocarbon were fed, can have but little relation to the therapeutic administration of mineral oil in large doses. It is well known that, when given in large quantity, mineral oil is chiefly excreted unaltered in the feces with an accompanying diarrhea. In the present experiment, in which the dosage was small and absorption excellent, no diarrhea at all was noted.

#### EXPERIMENTAL

$\Delta^1$ -Nonylenic acid was prepared by the condensation of malonic acid with heptaldehyde in pyridine solution and subsequent decarboxylation, according to Harding and Weizmann (8). The product was purified by distillation *in vacuo* and precipitation of the barium salt from ethanol. The regenerated free acid was redistilled, b.p. 169–175° at 30 min. pressure.

31.2 gm. of nonylenic acid were shaken with 500 mg. of platinum oxide (9) in an atmosphere of deuterium at room temperature and slightly less than atmospheric pressure. Deuterium uptake was rapid, and, after about 3 hours, the theoretical amount of gas had been consumed. Platinum was removed by filtration of the product in methanol solution and the product isolated by evaporation of the filtrate. The yield of crude  $\alpha,\beta$ -dideuterio pelargonic acid was 31.4 gm. Deuterium content, 9.1 atom per cent excess.

The electrolysis of the pelargonate was carried out essentially according to Petersen (10). To 20 ml. of ethanol + 25 ml. of water were added 5 gm. of  $K_2CO_3$  and 5 ml. of deuterio pelargonic acid. This mixture was electrolyzed between a platinum gauze anode and a copper cathode at about 1.5 amperes. The temperature was maintained at about 50° by use of an immersed "cold finger." At half hour intervals 5 ml. quantities of pelargonic acid were added until a total of 25 ml. had been introduced. Ethanol was also added from time to time to reduce foaming. The electrolysis was allowed to run 8 hours in all, during which time a pale yellow supernatant layer appeared.

The hydrocarbon was extracted from the reaction mixture with petroleum ether that had previously been purified over concentrated  $H_2SO_4$ . After evaporation of the solvent, the oily residue was stirred with concentrated

H<sub>2</sub>SO<sub>4</sub> at 100° for 1 hour, and from the resulting black tar the paraffin fraction was reextracted with petroleum ether, dried over KOH, and distilled *in vacuo*. The distillate was again purified by a second treatment with concentrated H<sub>2</sub>SO<sub>4</sub>, followed by a second distillation. The final yield, after this exhaustive purification, was 2.6 gm. M.p. 18°, deuterium content 9.7 atom per cent excess. On the basis of the deuterium analysis of the pelargonic acid, if it be assumed that no exchange had taken place during electrolysis, the expected deuterium content of the hexadecane would be 9.6 atom per cent. Because of difficulties inherent in carbon-hydrogen determination on samples rich in heavy hydrogen, a sample of hexadecane prepared and purified in an identical fashion, with the same melting point but devoid of isotope, was subjected to elementary analysis.<sup>2</sup>

C<sub>16</sub>H<sub>34</sub> (226.4). Theory, C 84.9, H 15.1; found, C 84.9, H 15.2

The feeding experiment was conducted precisely as in a previous report. To 186 gm. of basal diet already described (4) were added 12.5 gm. of butter and 1.5 gm. of deuterio hexadecane, the whole being homogenized in petroleum ether which was subsequently evaporated off. This diet was fed *ad libitum* to two growing male rats, average weight 94 gm., and was entirely consumed after 9 days. During this interval the rats showed an average weight gain of 31 gm.

The animals were killed and the lipids of the tissues and excreta isolated as in previous experiments (4, 5). The gastrointestinal tracts and feces were pooled and worked up together, and the livers were also investigated independently. Preliminary separation of the unsaponifiable fraction of the liver yielded too little material for isotope analysis, and it was therefore pooled with the corresponding fraction from the carcasses. To insure against contamination by isotopic hydrocarbon, the carcass fatty acids, after initial isolation, were redissolved in alkali and washed with ether. When isolated a second time, no significant alteration in deuterium content of the fatty acids had occurred. The separation of solid and liquid fatty acids was carried out as before (4), and the lead salts of the solid fatty acids were recrystallized from hot ethanol prior to their decomposition.

The unsaponifiable fractions were separated into alcoholic and non-alcoholic portions by refluxing with twice their weight of succinic anhydride in pyridine for 1 hour, dividing the reaction mixture between ether and dilute aqueous HCl, washing well with water, and then extracting the succinic acid hemiesters from the ether layer with Na<sub>2</sub>CO<sub>3</sub> solution. From the ether layer, the non-alcoholic fraction, which should contain any hydrocarbon that was present, was recovered by evaporation of the solvent. The aqueous layer, on reacidification and extraction with fresh ether, gave

<sup>2</sup> The elementary microanalysis was carried out by Mr. W. Saschek.



the succinic acid hemiesters of the hydroxy compounds present in this fraction.

All remaining operations were carried out as described in previous reports (4, 5).

#### SUMMARY

n-Hexadecane has been prepared containing an excess of deuterium, and this material has been fed to rats for a period of 9 days.

At the level of feeding employed, 83 mg. per rat per day, hexadecane is very efficiently absorbed from the gastrointestinal tract and partially deposited as such in the tissue lipids.

Much of the absorbed hexadecane was found to have been oxidized to fatty acid in the body, apparently largely in the liver.

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# LIVER GLYCOGENESIS AND FASTING IN THE RAT

## THE EFFECT OF GLUCOSE FEEDING ON THE WATER BALANCE

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Evidence was presented recently (1) that the process of glycogenesis when initiated by feeding glucose may result in an increase of as much as 32 per cent in the non-glycogen liver solids. This evidence is based upon a recalculation of the data of MacKay and Bergman (2). The purpose of the present work is to seek further evidence in order to test this conclusion. It appears that the problem is related to the mechanism of glycogenesis.<sup>1</sup>

### Methods

Male albino rats were used. Five of the groups of rats were approximately 100 days old and two of the groups from an entirely different colony were 160 days old.

The methods of analysis have been reported (1). The stock diet and anesthetic were the same as used by Guest (4). In these experiments considerable care was taken to exsanguinate the livers by pressing between filter paper. They were then frozen and weighed in an insulated container to  $\pm 5$  mg. On the other hand, in previous work reported, most of the blood was withdrawn through the heart before removal of the liver.

The livers were analyzed separately for water, glycogen, protein ( $N \times 6.25$ ), and lipids. Ash determinations were made upon mixtures of equal weights of fat-free dry solids. The mean values of the percentages of each component are given in Table I. Also given is the precision<sup>2</sup> of the means of certain measurements as  $\epsilon_M$ . An index of the accuracy attained is

<sup>1</sup> Another phase of the glycogenic process was investigated. The work of Deysach (3) on a sphincter mechanism in the liver suggests that circulatory differences may exist during glycogenesis as compared with fasting. An attempt was made to determine these differences. Hemostats were quickly applied to both the portal and hepatic blood vessels and the liver excised without loss of the contained blood. The latter was then pressed out on weighed filter paper. A comparison was made of livers with high and low glycogen. The results were inconclusive. In spite of efforts to avoid it, the mechanical pressure resulting from manipulation probably causes an abnormal blood distribution.

<sup>2</sup>  $\epsilon_M = \sqrt{\Sigma d^2 / (n - 1)n}$ .

furnished in the summation of the percentages of the individual tissue components.

No attempt was made either to force feed or to establish new feeding habits, as was done by Higgins *et al.* (5). It was found that following a 36 hour fast the rats ate a fairly uniform weight (about 10 gm.) of glucose paste.

Zero time was taken as 9 a.m. The body weight at this time is recorded as an initial value. It is the weight following a nocturnal period of feeding

TABLE I  
*Analytical Data*

Experiment No.	Dietary procedure	No. of rats	Age of rats	Average body weight			Liver weight	Average per cent in liver				
				Initial	After 36 hrs.			Water	Protein	Lipids	Glycogen	Ash
					fast	fast						
		days		gm.	gm.	gm.	gm.					
1	Fasted 36 hrs.	8	105	277 ± 4.3	247		6.89 ± 0.20	70.3	22.40	6.50	0.14	0.64
2	Fasted 36 hrs., fed 12 hrs.	8	110	269 ± 4.7	244		6.98 ± 0.11	70.2	18.5	5.86	4.95	0.63
3	" "	6	160	276 ± 7.0			9.45 ± 0.41	71.3	16.16	4.68	7.81	0.57
4	" "	7	110	268 ± 9.6			8.48 ± 0.50	69.4	16.43	4.43	8.90	0.64
5	Fasted 48 hrs.	7	110	271 ± 5.1	243	235	6.48 ± 0.24	70.4	22.01	6.03	0.31	0.64
6	Fasted 48 hrs., fed 12 hrs.	8	105	270 ± 3.7	244	240	6.82 ± 0.15	69.4	18.3	4.59	6.47	0.67
7	Fasted 24 hrs.	5	160	275 ± 7.0			8.29 ± 0.30	71.9	20.10	6.15	0.06	0.57
8*	" "	14	100	237			6.6 ± 0.24	71.2			0.38	
9†	Fed agar 24 hrs.	6	100	270			6.40	71.6	22.0	4.44	0.10	0.74

\* Unpublished data (1939) of Guest and McBride.

† These data have been published (1).

on the stock diet. During this period the rats eat intermittently beginning at about 8 p.m. Therefore, since no attempt was made to determine when they stopped feeding, what is referred to as a 24, a 36, or a 48 hour fast really represents a somewhat longer period. Guest (4) has shown that at about 9 a.m. the liver glycogen is at a high and uniform level, judging from the precision of the analyses.

The rats were selected so that the mean body weight is nearly the same for each successive group. All the rats were fasted and the experimental

groups were then fed glucose for 12 hours. According to the recalculated data of MacKay and Bergman, the maximum increase in non-glycogen solids occurred following a 48 hour fast and a subsequent 12 hour feeding period. Two of the experiments (Nos. 5 and 6) would have been more nearly comparable to those of MacKay and Bergman if the rats had been compared on the basis of their weight following a 48 hour fast. Instead, the author has chosen to adjust the liver weights to a uniform initial body weight of 270 gm. This facilitates comparison of groups fasted for different time periods and also comparison with some earlier data (1). This method of adjustment is based upon the linearity of the relationship of liver weight to body weight observed by Higgins *et al.* (5). However, in Experiments 5 and 6 both the initial and the final body weights at the end of a 48 hour fast are given. Therefore, adjustment can be made on either basis. The final adjusted values and the conclusions drawn by either method of calculation are much the same, because the fasting weight levels are the same for comparable groups within the probable limits of error.

A 48 hour fast may have a fairly severe physiological effect in view of the fact that Richter (6) found that rats survived about 4 days when deprived of food. For this reason the fast was reduced to 36 hours in certain experiments.

#### DISCUSSION

A comparison of the values for the non-glycogen solids in Table II shows that in no instance is there an accumulation of these solids when the glycogen reaches a high level. In fact, a loss of solids occurs which loss parallels that of the body weight and increases with an increase in the length of fast. In Table III are given the approximate values for the percentage loss of these solids with a variation in the length of fast. This loss occurs whether or not the liver has been synthesizing glycogen. The precision of the values of the non-glycogen solids indicates that the probability varies between 10 to 1 and 100 to 1 that these losses are significant. The data of Higgins *et al.* (5) show a loss under fasting conditions.

It had been hoped to employ the data in Table II to establish on a sounder basis the relationship of water to glycogen and possibly to protein. Judged both from the constancy of the non-glycogen solids and from the distribution of tissue components, there are two sets of experiments which appear to be ideal for comparison. Thus, Experiments 1 and 3 yield a ratio of glycogen to water of 1:2.7, which agrees with that previously reported. But, it necessitates a comparison of two groups which differ in age and come from different colonies. If the liver weights of these rats of unlike age had been compared on the basis of body area, as recommended by MacKay and Bergman (2), they might not appear to be comparable.

But, this need not alter the ratio observed. On the other hand, Experiments 4 and 5, which should be strictly comparable, yield a ratio of 1:1.9.

TABLE II  
*Distribution of Tissue Components*

The values are "absolute" weights adjusted to 270 gm. of initial body weight, measured in gm.

Experiment No.	Dietary procedure	Average weight		Average weight of component per liver					Ratio of glycogen to water
		Adjusted liver	Non-glycogen solids	Water	Protein	Lipids	Glycogen	Ash	
1	Fasted 36 hrs.	6.73 $\pm$ 0.12	1.98 $\pm$ 0.035	4.74	1.51	0.437	0.009	0.013	
2	Fasted 36 hrs., fed 12 hrs.	7.00 $\pm$ 0.11	1.73 $\pm$ 0.032	4.92	1.30	0.410	0.346	0.044	1:2.2
3	" "	9.24 $\pm$ 0.30	1.94 $\pm$ 0.061	6.58	1.49	0.432	0.720	0.053	1:2.7
4	" "	8.54 $\pm$ 0.35	1.86 $\pm$ 0.053	5.92	1.41	0.378	0.760	0.054	1:1.9
5	Fasted 48 hrs.	6.45 $\pm$ 0.18	1.89 $\pm$ 0.061	4.54	1.42	0.389	0.020	0.041	
6	Fasted 48 hrs., fed 12 hrs.	6.82 $\pm$ 0.09	1.65 $\pm$ 0.018	4.73	1.24	0.312	0.442	0.045	1:1.7
7	Fasted 24 hrs.	8.10 $\pm$ 0.21	2.30 $\pm$ 0.038	5.89	1.64	0.504	0.005	0.017	
8	" "	7.50	2.19	5.35					
9	Fed agar 24 hrs.	6.40	1.81	4.63	1.41	0.286	0.000	0.048	

TABLE III  
*Approximate Loss in Non-Glycogen Liver Solids*

	Duration of experiment	Approximate per cent of solids lost	Based upon experiments No.
	hrs.		
During fasting	24th to 36th	9.6	1 and 8
	24th " 36th	13.9	1 " 7
	36th " 48th	3.5	1 " 5
Following glycogenesis	36th " 48th	12.6	1 " 2
	36th " 48th	6.0	1 " 4
	48th " 60th	13.6	5 " 6

These ratios were calculated by use of a mean value for the ratio of non-glycogen solids of 1:2.4 from Experiments 1 and 5.

In Experiment 2 the glycogen level is low because the rats were fed only

one-half their normal intake of glucose. This may account for the relatively greater loss of solids observed in this instance.

It was noted that the rats drank very little water during the glucose feeding. This was particularly true in Experiment 6. Richter (6) also noted this low water intake in glucose feeding. Furthermore, most of the glucose-fed rats exhibited a marked distension of the stomach due to gas. There was no diarrhea as reported by Sinclair and Fassina (7). It appears that some rats cannot tolerate a high glucose intake, which in this instance approaches 3 per cent of the body weight. Such an intake is probably far enough in excess of the rate of absorption to result in a disturbance of the water balance.<sup>3</sup> Some evidence for this disturbance is furnished in the values for the percentage of liver water. These values are equal to approximately  $70.3 \pm 0.3$  per cent in both Experiments 1 and 5. Therefore, fasting from the 36th to the 48th hour does not alter the water content. But, feeding glucose for this interval, or from the 48th to the 60th hour, reduces it to approximately  $69.4 \pm 0.2$  per cent, as is shown in Experiments 4 and 6. This difference (0.9) is statistically significant. On the contrary, feeding glucose for this interval should increase the water content to more than 71 per cent, as it does in Experiment 3, in order that the ratio of glycogen to water remain 1:2.7. It has been shown in previous data (1) that under normal feeding conditions the percentage of water does not decrease with an increase in the glycogen content. It is not possible to attribute the difference in behavior of the rats in Experiment 3 to a solitary factor. They differ from the others as to age, strain, and stock diet. Sinclair and Fassina (7) have shown that the previous diet can alter the rate of glucose absorption. Perhaps some strains of rats exhibit cyclic liver changes under these conditions. This would explain the great increase in solids noted in the recalculated data of MacKay and Bergman.

Recently, MacKay and Drury (9) have shown that a large percentage of absorbed glucose is converted to fat. The present data indicate that, at least for the 12 hour period, the liver may not be involved in this process.

In Table IV are given the values of the ratios of protein and of lipids each compared to the non-glycogen solids. The fact that these ratios remain fairly constant in Experiments 1 to 6 inclusive indicates that these proteins and lipids constitute an essential part of the cell and do not represent storage materials. Evidently, when the liver loses solids following a 36 hour fast, it is whole cells which are lost. This is apparently not true of either the agar-fed or 24 hour-fasted rats. Of course, this does not take into account the cyclic variations studied by Higgins *et al.* (5).

It is of interest that the lipids here reported are composed of approxi-

<sup>3</sup> Holmes (8) found that glucose in amounts sufficient to disturb the fluid balance markedly would not generally elicit the drinking response in dogs.

mately 60 per cent of material precipitable by acetone from a petroleum ether solution. Since this fraction (presumably crude phospholipid) is quite hydrophilic, it may not be safe to assume that the cell lipids are not associated with water in some manner. This is in contrast with evidence discussed (1) concerning stored fat.

*Concerning Precision*—This type of experiment demands a high degree of quantitative comparability between the rats. The factor contributing most to the attainment of this comparability is probably the selection of the rats. The extent to which it was attained, as indicated by the precision, is reasonably good in view of the small number of rats employed. A comparison of the mean deviation of the mean for the unadjusted and adjusted liver weights indicates that adjustment improves the precision. It is thought that a better precision could be attained by selecting the

TABLE IV  
*Constancy of Composition of Liver Solids*

Experiment No.	Ratios	
	Protein to non-glycogen solids	Lipids to non-glycogen solids
1	0.762	0.221
2	0.750	0.236
3	0.766	0.222
4	0.757	0.203
5	0.743	0.204
6	0.750	0.189
7	0.714	0.218
9	0.778	0.158

rats to conform over a considerable time period to the standard growth curve of Zucker and Zucker (10). The precision could be further improved by adjusting the initial body weights for food intake.

The Fisher table (11) of values of *t* has been used in estimating the probability of significant differences.

#### SUMMARY

1. When rats are fasted for 36 or 48 hours and then fed glucose for 12 hours, there is no increase in the non-glycogen liver solids at high glycogen levels. On the contrary, there appears to be a loss of these solids which loss increases with an increase in the length of fast.

2. In some rats the feeding of large amounts of glucose may result in a disturbance in the water balance even though a diarrhea does not occur.

3. Evidence is offered to show that following a 36 hour fast it is essential cell constituents and not storage materials which are lost by the liver.

4. Possible alterations in the water balance and variations in the non-glycogen solids of the liver are complicating factors which make it difficult to calculate reliable values for the ratios of water to individual tissue components.

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# NON-OXIDASE NATURE OF KIDNEY "LACCASE"

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Owing to studies made in these laboratories on some plant oxidases, it was thought to be of interest to study the enzymatically active material observed by Cadden and Dill<sup>1</sup> to be present in cell-free kidney extracts. The observations made by these investigators led them to conclude that the material might be a polyphenol oxidase of the laccase type. Preliminary results obtained in the present study soon showed that the active principle is not a laccase, nor even a true oxidase, and for this reason it was decided not to pursue its study any further. Nevertheless, this active principle in the kidney extracts is of considerable interest and hence it was thought that the results obtained in the preliminary study might be worth recording in the literature.

Fresh ground swine kidneys were extracted overnight with 40 per cent acetone as described by Cadden and Dill. The extracted material was precipitated from the filtered 40 per cent acetone by the addition of 3 volumes of acetone. An aqueous solution of the precipitate thus obtained was then made 0.4 saturated with ammonium sulfate and the precipitate formed discarded. The filtrate thus obtained was then made 0.8 saturated with ammonium sulfate and the resulting precipitate taken up in water and dialyzed. The dialyzed solution was heated to 80°, cooled, filtered, and the precipitate which formed discarded. The resulting filtrate was adjusted to pH 4.5 and the active principle adsorbed to kaolin, followed by elution with 0.2 M secondary sodium phosphate, and finally dialyzed against distilled water. Satisfactory yields were obtained in each of the above steps. The solid matter in the final solution contained 15.6 per cent nitrogen and 0.03 per cent copper, indicating that it was a crude copper-bearing protein. The copper content increased as the purification progressed. This preparation catalyzed the aerobic oxidation of hydroquinone as reported by Cadden and Dill.

The enzymatic activity of the preparation was measured by means of a Warburg respirometer, with reaction mixtures consisting of 1 cc. of enzyme preparation, 2 cc. of 0.1 M phosphate buffer, yielding a pH of the final reaction mixture equal to 6.5, 50 mg. of hydroquinone, 20 mg. of sodium benzene sulfinate, and sufficient water to make the final volume equal to

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<sup>1</sup> Cadden, J. F., and Dill, L. V., *J. Biol. Chem.*, **143**, 105 (1942).

8 cc. The volume of the Warburg reaction flasks was approximately 45 cc. A control was found necessary for correcting for the autoxidation of the hydroquinone. The concentration of hydroquinone required for the optimum rate of oxygen uptake was found to be in excess of 0.1 M. The maximum rate of oxygen uptake was not reached until the experiments had been in progress from 10 to 20 minutes. Extending the time of the experiment beyond this length resulted in a gradual decrease in the rate of oxygen uptake. The addition of sodium benzene sulfinate to the reaction mixture not only increased the rate of oxygen uptake at low hydroquinone concentrations, but also resulted in a steady rate of oxygen uptake for at least 3 hours. Under the above conditions the best preparation caused an oxygen uptake of 7 c.mm. per minute per mg. of dry weight.

It was found that in the oxidation of the hydroquinone, catalyzed by the enzyme preparation, hydrogen peroxide was formed. This was demonstrated by means of catalase. Although it was found that the activity of the catalase was gradually destroyed when the latter was added to the reaction mixture, this inactivation was not so rapid but that considerable hydrogen peroxide could be decomposed. For instance, when catalase was added to the reaction mixture after 30 minutes of enzymatic oxidation of the hydroquinone, a rapid evolution of gas, presumably oxygen, occurred. The rate of oxygen uptake, however, gradually returned to the original value. The addition of hydrogen peroxide at this stage of the experiment caused no evolution of oxygen, showing that the return of the rate of oxygen uptake to its original value was due to the added catalase having become inactive.

In addition to hydroquinone, the kidney extract catalyzed the aerobic oxidation of catechol, homocatechol, and adrenalin. Its activity towards these substances was, however, less than towards hydroquinone. The extract was found to show no activity towards ascorbic acid, *p*-phenylenediamine, dihydroxymaleic acid, tyramine, *p*-cresol, pyrogallol, homogentisic acid, or 2,3-dihydroxynaphthalene.

When the enzyme preparation was heated at 100° for 15 minutes, the activity towards hydroquinone was diminished by 75 to 100 per cent. All the activity was lost when the enzyme solution was acidified to pH 2 and then brought back to pH 6.5. Potassium cyanide in concentrations of 0.004 M and 0.017 M inhibited the activity 17 and 62 per cent, respectively. Sodium azide in concentrations of 0.002 M and 0.01 M inhibited the rate of oxygen uptake 5 and 28 per cent, respectively. When a small amount of ascorbic acid (0.15 mg.) was added to the reaction mixture, a lag period of 90 minutes occurred before any appreciable oxygen uptake took place. The final rate of oxygen consumed, after the lag period was over, was the same as though no ascorbic acid had been added.

## DISCUSSION

Although the active constituent of the kidney extract is surprisingly heat-stable, the results given above strongly support the view that it is an enzymatically active protein. In particular may be cited (a) acid inactivation, (b) nitrogen content, (c) purification methods used, and (d) substrate specificity. On the other hand, the enzyme can hardly be classed as a true oxidase, *i.e.* belonging to the same class as cytochrome oxidase, tyrosinase, laccase, sweet potato catecholase, ascorbic acid oxidase, *etc.*, because it brings about only an incomplete reduction of the oxygen molecule resulting in the formation of hydrogen peroxide. In this respect the enzyme from kidney extract resembles more closely those of another class some of which are miscalled "oxidases," among which may be mentioned uricase, *d*-amino acid oxidase, and xanthine oxidase.



## THE AUTOLYSIS OF INVERTEBRATE TISSUES

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It has been well established that the gross mechanism involved in atrophy and autolysis is the same in all vertebrate tissues thus far examined (1). Proteolysis is accomplished by an enzyme complex called cathepsin. Further cleavage of the primary fragments is carried on by peptidases. While it is certain that there are large quantitative differences in the distribution of these enzymes in different tissues of the same species, and in the homologous tissues of different species, this field has not yet been adequately explored.

No systematic studies have been made to determine whether this or a similar mechanism is also to be found among the invertebrates, and very little is known concerning the phenomena of atrophy and tissue mobilization in this group. A number of isolated observations have been made, which indicate that an autolytic mechanism is present in invertebrate tissue. Thus Chen and Bradley (2) reported a comparative study of muscle autolysis which included squid and *Busycon* muscle, and indicated a low order of activity as compared with vertebrate muscle. Bishop (3) showed that the larval "fat body" of the honey-bee undergoes disintegration during pupation and showed an increased proteolytic rate at that time. Many studies have been made upon the *digestive* enzymes of invertebrates based upon their extraction from tissues. The interpretation of these results must remain doubtful, since the proteinases reported may be autolytic rather than food-digesting in function. In general also, these older observations were made before accurate control of the pH was possible and before the phenomenon of activation had been discovered. It will not be profitable therefore to review this literature. The more recent and accurately controlled experiments on invertebrate digestion have been reviewed recently by Vonk (4). In a number of investigations cathepsin is reported as a digestive enzyme, but its possible function in an autolytic rôle is not considered (5). The present study was planned therefore as a preliminary survey of the autolytic behavior of such invertebrate tissues as could readily be obtained in sufficient quantity at Madison, Wisconsin, and Woods Hole, Massachusetts.

Our sampling of the phyla is obviously limited. The two annelids examined were the common earthworm *Lumbricus terrestris* and *Nereis virens*,

representing respectively a land form and a sea form. Among the Mollusca we have studied the marine lamellibranchs *Venus mercenaria* and *Peecten magellanicus* and the fresh-water forms *Lampsilis*, *Anadonta*, and *Lasmigona*; the marine gastropod *Busycon* was studied and the cephalopod *Loligo pealii*. *Homarus americanus* and the large edible shrimp *Pencus setifera* represent the Crustacea. *Limulus polyphemus* represents the Arachnids.

In the case of the worms and the lamellibranchs the whole organism was used. It is obvious that such digests represent the proteinases of the various tissues together with such digestive enzymes as may normally be secreted into the intestinal tract. The results obtained with *Nereis*, *Lumbricus*, and the lamellibranchs can only be provisionally interpreted, since it was not possible to separate the digestive system and its enzymes from the other tissues. In the other forms we were able to study single tissues, just as has been done with vertebrates.

#### EXPERIMENTAL

Digests were prepared and set up as described in a previous paper (1). Hemoglobin was added to some digests as a foreign protein of known fragility to supplement the tissue proteins themselves, of whose availability as substrate little is known. The presence of this extra substrate may serve to disclose a proteolytic enzyme even when the cell proteins themselves are not fragmented. Digests were maintained at the initial pH by frequent readjustments during the first 3 days of autolysis with the glass electrode. The pH levels used were 2.0, 3.0, 4.0, 5.0, 6.0, and 7.5. In the case of *Limulus* eggs intermediate levels were also used. Samples were precipitated by trichloroacetic acid of 5 per cent final concentration, and digestion measured by the increase in soluble nitrogen in the filtrates and by the increase of the tyrosine color reaction. In some digests we were unable to avoid the development of white precipitates with the tyrosine reagents, but, as they can be quickly removed by centrifugation and do not carry down the color compound, no inaccuracies of color measurement were involved. All digestions were carried on at 38° unless otherwise specified. In some cases the activating effect of cysteine and the inhibiting effect of  $\text{KIO}_3$  were tried, to discover further similarity to vertebrate tissue proteinases.

*Nereis*—The whole organism was ground fine and homogenized. The digestion curve represents a composite of tissue proteinases plus those from the digestive tract and glands. From the shape of the curve (Fig. 1) it is clear that this mixed material contains proteinases predominantly of the tryptic type, with optimum pH at about 7 to 8. The fact that digestion goes on at pH 2 to 4, however, indicates the presence of proteinase

active in this more acid range. A very similar curve is given by hog pancreas under identical conditions, when both trypsin and cathepsin are known to be present (6). Cysteine and  $\text{KIO}_3$  produced little activation or inhibition respectively. Raw hemoglobin is digested best at pH 8, which indicates that an enzyme is present not identical with mammalian trypsin, to which undenatured hemoglobin is resistant. For the present, the evidence indicates an active tryptase and a less active proteinase with an optimum in the region of pH 3 to 5.

*Angleworm*—The entire organism was used. Proteolytic activity (Fig. 2) is shown by this composite material between pH 3 and 7.5, with a definite optimum at pH 5. This also suggests several overlapping proteinases or a generalized enzyme active over a wide range.

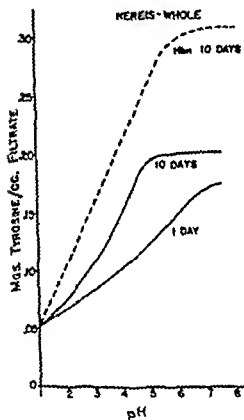


FIG. 1

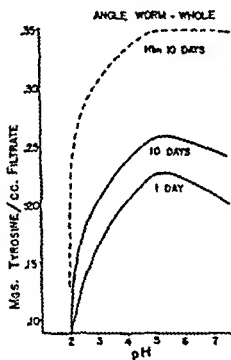


FIG. 2

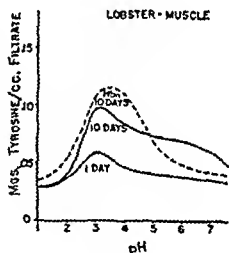


FIG. 3

*Lobster Muscle*—Material was obtained from the tail and leg muscles free from other tissues or secretions. Fig. 3 shows a low order of proteolytic activity with only a small digestion of hemoglobin in the 10 day period. A pH of 3 is optimum, with some digestion proceeding between pH 4 and 6. Cysteine did not activate digestion, though  $\text{KIO}_3$  gave significant inhibition. This probably indicates complete activation of the system by the sulfhydryl compounds already present in the tissue. The failure of hemoglobin to be digested in the neighborhood of pH 6, while the muscle proteins are digested, seems to indicate a tryptase superficially not unlike mammalian trypsin. It is evident that lobster muscle protein can only be mobilized slowly for general use by this organism.

*Lobster Digestive Gland*—The hepato-pancreas of the lobster is believed



to be a true digestive organ secreting into the gastrointestinal tract. Our study of this tissue was preliminary and because of lack of time and material was adequate only to show that proteolysis at pH 4 is so rapid as to be nearly complete in 24 hours, with much greater liberation of tyrosine than in most of the invertebrate tissues we have examined. At pH 7.5 digestion is also quite good, though less rapid and complete. Cysteine shows a slight activation at pH 4 (Table I).

*Shrimp Muscle*—Refrigerated shrimps were obtained and the caudal muscle dissected free from the intestine and shell. The figures indicate a small total digestion, with a sharp maximum at pH 3 and no significant digestion at pH 5 and above, which is essentially the same as that for lobster muscle. Added hemoglobin was not digested at any pH tried.

*Venus*—Whole living clams were ground fine. The digestive mass, or midgut gland, makes up about 60 per cent of the entire soft tissues, and is richly supplied with the phagocytic cells which carry on the digestion of the

TABLE I  
*Lobster, Digestive Gland*

	pH	Mg. tyrosine per 1 cc. filtrate				
		0 day	1 day	3 days	5 days	10 days
Control.....	7.5	0.09	0.27	0.29	0.30	0.33
" .....	4.0	0.09	0.40	0.43	0.49	0.49
" and Hb.....	4.0	0.09	0.60	0.63	0.63	0.63
" " cysteine....	4.0	0.09	0.42	0.49	0.53	0.53

fine particles accepted as food by this form. According to Yonge (7) digestion of food proteins is accomplished by these wandering cells in the lamellibranchs and no proteolytic enzymes are secreted into the gut.

Two enzymes are clearly indicated (Fig. 4). The predominant one shows a maximum autolysis and digestion of hemoglobin at pH 3 to 4, with a great deal of activity even at pH 2+. This type of enzyme we have found characteristic of molluscan tissues. It resembles vertebrate cathepsin grossly and is strongly activated by cysteine and inhibited by KIO<sub>3</sub>. A second maximum, much smaller, is found at pH 6.5, which suggests a trypsin.

*Anadonta and Lampsilis*—These medium sized, thin shelled, fresh-water clams are abundant in the Madison lakes and were obtained in the fall just before the winter resting period. The whole animals were used, and the curves of autolysis are very similar to those of the sea-clam just described. Digestion of the clam tissue is very rapid and is complete in 5 days. No trypsin was found.

*Lasmigona*, a thick shelled clam often weighing 400 gm. or more, gives an identical digestive pattern.

*Pecten Muscle*—The large adductor muscle of this bivalve is readily obtained refrigerated in the market. While not perfectly fresh, it had been kept frozen and probably represents the living muscle fairly well (8).

Fig. 5 indicates a very small amount of an enzyme similar to that of the bivalve just reported. Hemoglobin is digested slowly, which confirms the impression of a very small amount of enzyme present. Cysteine produces a slight activation and  $KIO_3$  a slight inhibition. This tissue is strikingly stable. Although it makes up about half the total soft tissue of the animal, it evidently cannot be easily or rapidly mobilized for use by the organism as a whole. It is rich in glycogen, and acts as a fuel reservoir. Functionally it is rather inert as a contractile mechanism, though it is capable of making a series of rapid contractions by which the scallop occa-

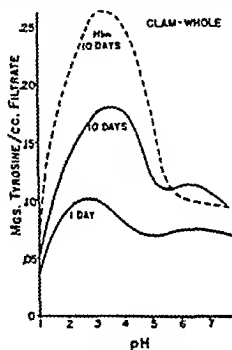


FIG. 4

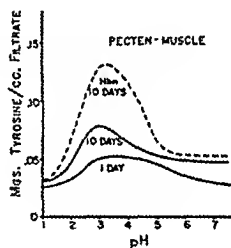


FIG. 5

sionally moves about. In this respect it is the most active bivalve muscle that we have studied.

*Squid Tentacles*—The squid is the most active of the invertebrates examined, and the most highly developed. It is in constant motion and capable of bursts of great speed for short periods. It cannot support a sustained high speed effort, however, and is quickly fatigued to the point of helplessness. It is said not to survive long in captivity because of its constant activity and the difficulty in providing food which it will take.

The tentacles were removed and immediately washed to remove salivary secretion ejected from the mouth in the premortal struggles. We suspect that the tryptic enzyme found in these digests was due nevertheless to such contamination.

Autolysis is much more rapid and extensive than in other molluscan muscles we have examined. In Fig. 6 the characteristic sharp peak is shown at pH 3.

*Squid abdominal muscle* shows almost exactly the same digestion curve

(Fig. 7) as that of the tentacles except for the fact that there is less evidence of a tryptase.

*Busycon*, *Pedal Muscle*—The pedal muscle of this large gastropod makes up nearly 50 per cent of the total animal, exclusive of the shell. When contracted it is a firm hard mass which can be easily obtained free from contaminating tissue or fluid. It is very difficultly dispersed, however, even by repeated grinding and homogenizing, and remains unsatisfactory for sampling. In the more acid digests the particles soften somewhat and become semigelatinous. There is little or no gross evidence of digestion under any conditions. Because of the difficulties of sampling, the individual results are subject to large errors. However, from many experiments, the curves shown in Fig. 8 are believed to be characteristic and reproducible.

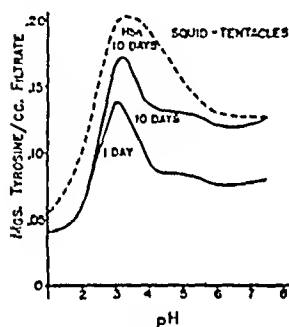


FIG. 6

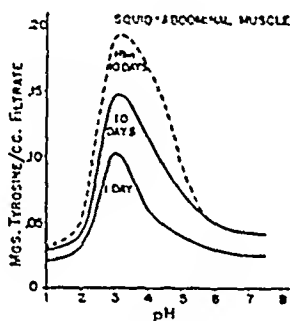


FIG. 7

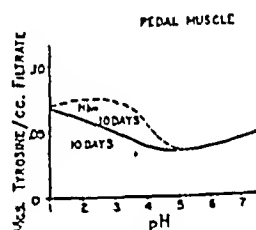


FIG. 8

There is very little autolysis in 10 days at any pH. There appears to be slight digestion in high acidity, which falls off to a minimum at pH 5 and shows a slight increase at pH 7.5. The slight increase of reactive tyrosine compounds is so small as to make it doubtful whether we are measuring autolysis. There does seem to be a slight digestion of hemoglobin between pH 1 and 3, which is probably evidence of a trace of active proteinase present. Altogether the pedal muscle of *Busycon* resembles most closely the inert adult connective tissues of the mammal, such as cartilage, yellow elastic and collagenous masses of ligaments, and tendons. Functionally the muscle is very slow moving and stands at the bottom of our muscle series so far as active contraction is concerned. It is well supplied with glycogen, like most molluscan muscle, but can hardly contribute protein significantly for general use of the organism. We have no data to indicate whether atrophic changes ever do occur in this muscle during starvation.

*Albuminiferous Gland*—A few ripe females were obtained in which the egg capsule-secreting gland was well developed and ready for capsule

formation. When ground, this tissue forms a stiff gummy mass rather hard to handle satisfactorily. It is loaded with the precursor of the protein that forms the insoluble string of "purses" within which the embryos develop. It was unlike any other glandular tissue we have examined, in that autolysis was very slight, with a maximum at about pH 3, of the same order as that found in the pedal muscle. There was considerable digestion of hemoglobin, however, at this pH, which indicates that the enzyme is not lacking but that the tissue proteins themselves are not available for cleavage by it (Table II).

The egg capsules themselves are composed of a highly insoluble protein, which is not digested in pepsin-HCl or active trypsin solutions. The gland presents, therefore, some interesting problems in protein transformation

TABLE II  
*Busycon*, Capsule Gland

	pH	Mg. tyrosine per 1 cc. filtrate			
		0 day	3 days	5 days	10 days
Control	1.7	0.023	0.032	0.040	0.043
	3.0	0.023	0.055	0.057	0.058
	3.5	0.023	0.042	0.047	0.050
	4.5	0.023	0.026	0.026	0.037
	6.3	0.023	0.027	0.037	0.037
	7.5	0.023	0.031	0.042	0.041
" and Hb	1.6	0.025	0.058	0.064	0.072
	3.0	0.025	0.100		0.115
	3.3	0.025	0.100	0.135	0.155
	4.2	0.025	0.055	0.058	0.058
	7.5	0.025	0.033	0.045	0.054

from the viscous precursor to the insoluble final product of secretion, after it has come in contact with sea water.

*Digestive Gland*—The digestive gland of *Busycon* shows very strong proteolytic activity. With the exception of the hepato-pancreas of the lobster and the eggs of *Limulus*, it is the most active invertebrate tissue we have found. It shows the characteristic sharp peak at pH 3 common to all molluscan tissues we have examined, with a secondary low optimum around pH 6 to 7, indicating a tryptic enzyme present in small amount (Fig. 9). The gland itself has been reported to contain no trypsin-like enzyme (9), while the salivary glands do. It is believed to be absorptive rather than secretory in function. The low tryptic activity shown here is probably due to the presence of some salivary secretion in the absorbing diverticulae of the gland, which could not be entirely excluded from the material.

*Limulus polyphemus*—Several large females were secured heavily loaded with mature eggs. The latter could be separated easily from ducts and ovarian tissue, washed, and obtained uncontaminated. The muscle was dissected free from other tissues and from blood and hepato-pancreas.

*Muscle*—Muscle tissue autolyzes with a sharp optimum at pH 3. Digestion between pH 2 and 3 is rapid and nearly complete in 3 days. There is no digestion at pH 6 and 7.5, and only a very small proteolysis proceeding slowly at pH 5. Between pH 3 and 5, notably at pH 4, digestion proceeds slowly, but finally attains nearly maximum proteolysis (Fig. 10). Hemoglobin is digested best at pH 3, but not at all at pH 2 or 5. The sharpness of the digestive curve is the striking feature of this tissue.

*Hepato-Pancreas of Limulus*—The brown masses of this tissue can be readily freed from oviducts and eggs. It is homogenized to a smooth

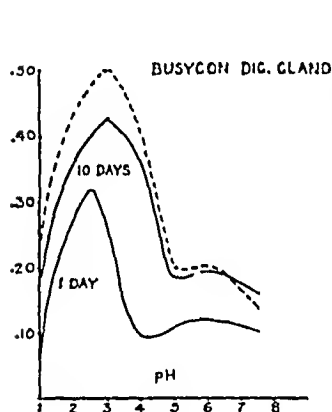


FIG. 9

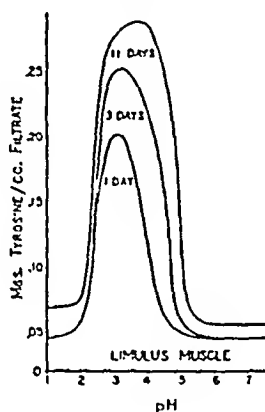


FIG. 10

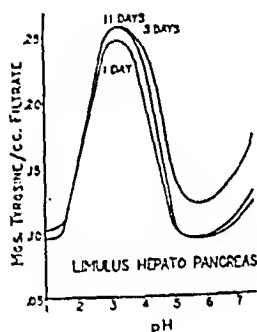


FIG. 11

suspension susceptible of accurate sampling. Two optima were found, one at about pH 3.5 which indicates a cathepsin, the other at pH 7.5 or more which indicates a tryptase (Fig. 11).

*Eggs*—The opaque gray-green eggs, with a diameter of about 2 mm., are obtainable in quantity from the gravid females during the early summer. By using screens it is possible to obtain the eggs entirely free from contaminating material. They are not easily ruptured even by fairly rough handling in the process of separation and cleansing. They are ground up easily into a smooth creamy suspension and are quickly homogenized to a uniform fine dispersion except for the tough egg membranes. The dispersions are so uniform and the particle sizes so small that digests may be sampled with the regular fine tipped pipettes.

A preliminary autolytic series showed that digestion was extraordinarily rapid and complete, and that activation by cysteine was considerable, as

was inhibition by  $KIO_3$ . A second series was set up with eggs obtained from two large females and maintained at a room temperature of about  $24^\circ$  in order to slow the digestion rate. After 12 days the digests were transferred to the warm room at  $30^\circ$ . The intermediate curves are therefore not strictly comparable to the others presented here, though the final digestion figures are.

As Fig. 12 shows, there is no digestion at all at pH 2. At pH 2.5 digestion is rapid. In the early hours of digestion the optimum is sharp and close to pH 3. It gradually shifts to pH 3.5 as the process continues, and when final equilibrium is approximated the optimum zone is broader and extends nearly to pH 4. Digestion is almost zero at pH 5, and there is no evidence

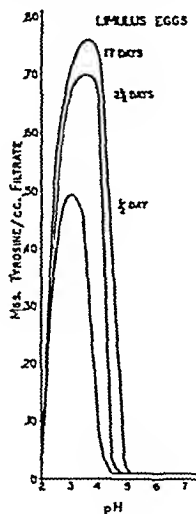


FIG. 12

of proteolysis beyond that point. It is evident that very small shifts in the H ion in the neighborhood of pH 4.5 will cause extraordinarily large differences in the amount of protein mobilized and in the speed of this mobilization. Cysteine increases the digestion rate and extent at all pH levels where digestion normally occurs.  $KIO_3$  inhibits strongly, at the pH optimum, but digestion continues slowly. The results are strikingly similar to the digestion of mammalian liver for example, except that the curves are smoother, narrower, and more sharply defined. This is due in part to the fact that this material can be prepared in a homogeneous suspension which lends itself to accurate sampling and analysis. The symmetry of the curves and the narrow pH range suggest that a single protein consti-

tutes the substrate, or, if more than one protein is concerned, at least that isoelectric points and fragility of the proteins present are much alike.

The significance of the autolytic mechanism in the eggs appears to be clearly a provision for the mobilization of stored protein into amino acids for the synthesis of new tissue protein in the developing embryo.

#### DISCUSSION

From the results of the limited sampling of invertebrate tissues here presented, it appears probable that an autolytic mechanism exists throughout the invertebrate field, superficially at least like that found in all vertebrate tissues. Cleavage is initiated by a proteinase whose maximum activity is found to be close to pH 3, and whose effective range is from pH 2.5 to 5. In some tissue there may be additional proteinases which act best near the neutral point or in slight alkalinity. The amount of activity, or enzyme present, and the availability of the tissue proteins vary greatly. This variation finds its counterpart in mammalian tissues also. In comparing muscles of the several species of molluscs studied we find evidence that functional activity of the contractile mechanism correlates with its autolytic activity, as appears to be the case with vertebrate muscle.

As a general rule we have found invertebrate tissue proteinase to be susceptible of activation by cysteine and of inhibition by  $\text{KIO}_3$ . In some instances there is no activation, but the inhibitory effect of the oxidant is significant. In these cases it seems probable that the enzyme is already fully active when the tissue mince is prepared, and the brilliance of the nitroprusside reaction in such tissues is in harmony with this explanation.

Our tentative conclusion therefore is that a catheptic type of proteinase is widely distributed in invertebrate tissues and initiates the autolytic mobilization of the tissue proteins. Its optimum appears to be somewhat more acid than that found in vertebrates. In certain forms we believe the autolytic mechanism also functions as the digestive proteinase—specifically in the lamellibranchs in which a true proteolytic secretion into the gut is doubtful and in which the primitive device of phagocytosis appears to be the only provision for securing amino acids from the food particles.

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# FATTY ACID OXIDATION BY LIVER ENZYMES

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The enzymes which oxidize lower saturated fatty acids are difficult to study because of their great instability. It has been found (1) that butyric acid can be oxidized by liver tissue even after the cell structure has been destroyed. The oxidizing system is inactivated more rapidly in the absence of oxygen, and the addition of fumarate increases the rate of oxidation.

An enzyme preparation from rat liver, which oxidizes higher fatty acids, was described by Lang (2). Adenylic acid acted as a coenzyme and the reaction product was the corresponding unsaturated acid (3, 4). Previous work has been reviewed by von Euler (5) and Lang (2).

Further work on the oxidation of butyric acid and of other acids by washed preparations of liver has revealed the identity of some of the components of the oxidizing system.

## EXPERIMENTAL

*Methods*—Butyric acid was estimated as previously described (1) after precipitation of the proteins with zinc sulfate and sodium hydroxide. Alkaline evaporation was omitted. Phosphate was estimated according to Fiske and Subbarow (6) after deproteinization with 1 volume of cold 5 per cent trichloroacetic acid. The phosphate liberated in 7 and 60 minutes by 1 N hydrochloric acid at 100° and by hypiodite, according to Lohmann and Meyerhof (7), was also estimated. Oxygen uptake was measured with Warburg manometers.

Adenylic acid was prepared as described by Lohmann (8). Cytochrome from muscle extract (9) was dried with 4 volumes of cold acetone and kept in a desiccator (about 10 per cent pure). Solutions in 0.1 M sodium chloride were prepared each day.

Experiments were carried out in Erlenmeyer flasks with shaking at 25° and in air.

*Inactivation of Enzyme System*—Homogenized liver tissue is inactivated more rapidly in the absence of oxygen (1). Before an attempt was made to purify the system, it was considered convenient to find out whether oxygen acted on the coenzymes or on the enzyme proper. The experiment in Table I shows that the preparation which becomes inactive after 10 minutes anaerobically is reactivated by a heated extract of liver.



If more time is allowed to elapse, the enzyme becomes inactive with or without oxygen and is not reactivated by the heated extract. However, this length of time is sufficient to obtain a partial purification if the temperature is kept low.

*Preparation of Enzyme*—Guinea pig liver removed immediately after death was washed with ice-cold water, cut with scissors, and again washed. The tissue was then transferred to a stainless steel tube closed at one end ( $2.5 \times 25$  cm.) containing 4 volumes of water. A steel ball (diameter 0.5 mm. smaller than the tube) fixed at the end of a rod was forced into the tube and moved vertically. After 60 double excursions of the ball an adequate rupture of the cells was obtained. The whole procedure was carried out at the lowest possible temperature without freezing. After 0.1 volume of 1 M magnesium chloride was added, the preparation was

TABLE I  
*Influence of Oxygen on Stability of System*

Rat liver was homogenized in 1 volume of 0.1 M sodium chloride and filtered through muslin. One-half was kept at 25° in oxygen, the other half in a test-tube without oxygen during 10 minutes. 2 ml. portions were added to Erlenmeyer flasks containing 0.2 ml. of 1 M fumarate + 2 ml. of water or heated liver extract (5 minutes at 80° in 1 volume of water). Total volume, 4.2 ml.

Treatment of homogenized liver	Butyrate disappearance
	<i>micromoles</i>
Kept in O <sub>2</sub> .....	8.7
" " " + heated extract.....	11.3
Without ".....	0.6
" " + heated extract.....	10.7

filtered through muslin and centrifuged 5 minutes at 6000 R.P.M. in a cooled centrifuge. The precipitate was suspended in 3 volumes of water, homogenized, and 0.1 volume of magnesium chloride was added. The suspension was again centrifuged and the washing repeated twice more. The precipitate after the fourth centrifugation was suspended in 3 to 4 volumes of 0.1 M fumarate or 0.03 M sodium chloride according to the experiments. The whole procedure lasted about 40 minutes. The activity and degree of purity of the preparation depend on the degree of cell rupture, on the volume of liquid used in washing, on the temperature, and on the time the preparation remains suspended in pure water.

If the cell rupture is insufficient, a clean preparation is obtained only after many washings. If it is excessive, the preparation becomes inactive.

The suspension of the precipitate in water produces not only hemolysis and the removal of many cell substances but also produces inactivation in

a few minutes. To prevent this inactivation only 20 to 40 seconds should elapse between the suspension in water and the addition of the magnesium chloride solution. The addition of salts preserves the activity and makes the centrifugation easier. Magnesium chloride is better than sodium chloride because it produces a greater insolubility of some active components.

At 0° the enzyme lasts 2 to 3 hours, less at a higher temperature. Freezing produces complete inactivation.

Owing to all these factors and to the variable composition of the liver, the resulting preparation varies somewhat in activity and purity. With one or two more washings a cleaner preparation is obtained, but the danger of inactivation is increased. Even with the procedure as described inactive preparations are sometimes obtained.

TABLE II  
*Components of Oxidation System*

Disappearance of butyrate produced by 2.5 ml. of liver enzymes + 0.2 ml. of 1 M fumarate + 0.2 ml. of M/15 phosphate buffer of pH 7.7 + 0.1 ml. of 0.1 M magnesium chloride + 0.5 mg. of cytochrome *c* preparation + 1 mg. of adenylic acid + 17.3 micromoles of butyrate. Total volume, 6 ml.; 90 minutes at 25° in air.

	Butyrate disappearance	
	micromoles	micromoles
Complete system.....	8.2	8.9
No phosphate.....	1.9	2.6
" fumarate.....	3.0	2.6
" cytochrome <i>c</i> .....	-1.2	-1.2
" adenylic acid.....	-0.9	-1.0

*Components of Oxidation System*—The washed tissue is completely inactive by itself, but will oxidize butyrate when a heated extract of liver, kidney, or heart is added. The fractionation of these heated extracts led to the identification of several components, as is shown in Table II.

In the absence of phosphate or fumarate the system showed a slight activity, but not comparable with that of the complete system. In the absence of cytochrome or adenylic acid the preparation was completely inactive.

In these experiments the enzyme was prepared with magnesium chloride. In other experiments in which sodium chloride was used in the preparation it was found that the system was inactive in the absence of magnesium or manganese.

The optimum concentration of phosphate appears to be about 0.01 M, although the results varied somewhat with different preparations. With

0.002 M a slightly smaller disappearance of butyrate was obtained. This was the amount used in the experiments in which phosphate was estimated. The optimum concentration of fumarate under the same conditions was found to be 0.03 M. With greater or smaller concentrations there was a decrease in activity. Malate proved to be much less active than fumarate in increasing the rate of oxidation of butyrate, and succinate was inactive.

With varying amounts of adenylic acid the activity was as follows:

Adenylic acid, mg.....	0	0.2	0.5	1	2	4
Butyrate disappearance, micromoles	0	5	9.9	11	11.7	11.7

Adenylic acid can be replaced by adenyly pyrophosphate.

Table III shows the results of an experiment in which the oxygen uptake was also measured.

TABLE III  
*Components of Oxidation System*

The composition of the system was as in Table II. The oxygen uptake was measured in 3 ml. aliquots and calculated for 6 ml. in micromoles of oxygen.

	Oxygen uptake		Butyrate disappearance
	With butyrate	No butyrate	
	micromoles	micromoles	micromoles
Complete system.....	45.0	33.2	9.5
No fumarate.....	2.6	10.0	1.0
" phosphate.....	30.2	21.2	3.6
" adenylic acid.....	10.8	11.8	-0.1
" cytochrome.....	5.0	4.3	0.1

The highest oxygen uptake was found in the complete system with butyrate. However, even without butyrate the oxygen uptake is rather high, owing presumably to the oxidation of fumarate. The relation between oxygen uptake (after subtraction of the blank) and butyrate disappearance is 1.2; i.e., about 1 molecule of oxygen per molecule of butyrate. The butyrate oxidation in the absence of added phosphate was probably due to the impurity of the enzyme which contained 0.6 micromole of inorganic phosphate.

*Oxidation of Other Acids*—The oxygen uptake of some compounds related to butyrate was measured with and without adenylic acid (Table IV).

Butyrate and crotonate are the most rapidly oxidized. The latter also needs adenylic acid. Isocrotonate also gave an increase in oxygen uptake but smaller than did crotonate; the sample used was not pure and may have contained some crotonate; nevertheless the experiment shows that it is oxidized more slowly than crotonate.  $\beta$ -Hydroxybutyrate gave a small increase even in the absence of adenylic acid.

TABLE IV

*Influence of Adenylic Acid on Oxidation of Butyrate and Related Compounds*

Oxygen uptake in 30 minutes at 25° by 3 ml. of the complete system as in Table II with or without adenylic acid. Substrate concentration 0.003 M.

Substrate	Adenylic acid	Oxygen uptake microliters
None.....	-	42
".....	+	101
dl- $\beta$ -Hydroxybutyrate.....	-	53
".....	+	126
Crotonate.....	-	24
".....	+	185
Isocrotonate.....	-	23
".....	+	139
Butyrate.....	+	172

TABLE V

*Oxidation of Various Acids*

Oxygen uptake in 30 minutes at 25° of 3 ml. of the complete system as in Table II with different acids (concentration, 0.003 M). The figures given represent the uptake in microliters after the uptake with no substrate which is given in the top row is subtracted. A negative sign indicates an oxygen uptake lower than with no substrate.

No substrate.....	83	94	102	155	107
Formate.....				-6	-11
Acetate.....			9	10	
Propionate.....	-5	-17			15
Lactate.....			7	6	
Pyruvate.....				22	-1
Butyrate.....	63	83	76	49	72
Isobutyrate.....		6		34	41
$\alpha$ -Bromobutyrate.....	-14				3
Valerate.....	50		80		
Isovalerate.....	2		12		30
Hexanoate.....		109			80
Heptanoate.....	58		99		
Octanoate.....	113	25			
Decanoate.....			-48	-141	
Dodecanoate.....			-80	-136	
Stearate.....		-25			5
Palmitate.....	-1	-66			
Oleate.....		-73	-84		

As Table V shows, the oxygen uptake was greatest with butyrate, valerate, hexanoate, heptanoate, and octanoate; less with isobutyrate and

isovalerate. Results with formate, acetate, lactate, pyruvate, propionate, and  $\alpha$ -bromobutyrate were doubtful, while decanoate, dodecanoate, stearate, palmitate, and oleate showed a decrease in the oxygen uptake.

*Changes in Phosphate*—As shown in Table VI, in the complete system without butyrate there occurs an uptake of inorganic phosphate. This uptake is very small in the absence of adenylic acid. In the absence of cytochrome or fumarate there is an increase in inorganic phosphate which is liberated from the enzyme preparation which contains about 7 micromoles per ml. of bound phosphate.

TABLE VI  
*Changes in Inorganic Phosphate*

The complete system was as in Table II. The results are given in micromoles.

	Initial	No butyrate	With butyrate	Butyrate disappearance
Complete system.....	15.6	6.2	11.8	14.6
No cytochrome.....	15.6	19.6	20.1	0.4
" fumarate.....	15.4	20.4	18.2	6.5
" adenylic acid.....	15.2	14.1	16.4	0.9

TABLE VII  
*Changes in Phosphate Fractions*

The complete system was as in Table II. The results are given in micromoles. Each figure is the mean of duplicate estimations not differing by more than 3 per cent.

Complete oxidation system	Inorganic P		P hydrolyzed in 7 min. in N HCl at 100°	P hydrolyzed in 60 min. in N HCl at 100°	P liberated by hypiodite	Titration with hypiodite*	Butyrate disappearance
	Initial	Final					
With butyrate.....	15.3	16.5	0.9	1.2	0	7.3	7.5
No butyrate.....	15.0	12.1	1.9	5.7	4.7	12.2	0

\* Corresponds to the iodine consumed by the samples in alkaline medium and calculated as pyruvic acid (6 equivalents of iodine per mole of pyruvic acid (7)).

When butyrate is present, the final amount of inorganic phosphate is larger than in the control with no butyrate. The difference is all accounted for by a greater formation of phosphopyruvic acid and of adenylyl pyrophosphate, as is shown in Table VII. Tests for labile phosphate of the phosphocreatine type (10) or of the acetyl phosphate type of Lipmann (11) invariably gave negative results. All these experiments have been repeated many times with the same results.

With low concentrations of fumarate or when none is added as in Table VI there occurs a smaller disappearance of butyrate, while the increase in inorganic phosphate does not occur.

With acetate,  $\beta$ -hydroxybutyrate, and acetoacetate no changes in inorganic phosphate occurred, while with propionate, valerate, hexanoate, octanoate, decanoate, and stearate an increase in inorganic phosphate was found as with butyrate.

*Inhibitors*—Fluoride produced a complete inhibition of the disappearance of butyrate at 0.01 M, and about 80 per cent at 0.002 M. The same inhibition was obtained with iodoacetate. Arsenate also acted as an inhibitor, as did malonate. The latter effect is unexpected, because in liver slices malonate increases the spontaneous formation of ketone bodies and does not inhibit the oxidation of fatty acids. Methylene blue acted as an inhibitor at 0.01 per cent and had no effect at lower concentrations.

*Oxidation Product*—Many experiments have been carried out in order to measure the amount of acetoacetate and  $\beta$ -hydroxybutyrate formed. Variable results have been obtained, but as a rule hardly any acetoacetate was formed and the amount of  $\beta$ -hydroxybutyrate corresponded approximately to the amount of butyrate which disappeared. The oxygen uptake corrected for the blank corresponded to about 1 molecule per molecule of butyrate. However, owing to the lack of specificity of the method and to the variable results, it is considered that further work is necessary before the results are published.

#### DISCUSSION

The preparation of enzyme systems which are as labile as that which oxidizes butyrate is important, because it is likely that many physiological reactions are catalyzed by such systems. The procedure which is described may be useful in studies on tissue metabolism even if it yields impure preparations only, and often inactive products.

It is interesting that some of the components of the system which have been identified (fumarate, phosphate, magnesium, and adenylic acid) are also involved in the oxidation of carbohydrates and their derivatives. Perhaps this will give some clue on the relation between fat and carbohydrate metabolism.

The oxidation appears to be in some way coupled with a phosphorylation. Inorganic phosphate, adenylic acid, and magnesium which are known components of phosphorylating systems are needed for the oxidation. The specific inhibitors of phosphorylations (fluoride, iodoacetate) also inhibit the oxidation of butyrate. The changes which occur in the distribution of phosphate are difficult to understand. In the absence of fatty acids, fumarate is transformed into phosphopyruvate or into a substance that gives the same reactions, as has been found by Kalekar (12), and adenylic acid becomes phosphorylated. When butyric acid is oxidized, hardly any phosphopyruvate or adenylyl pyrophosphate is formed. This does not appear to be due to an inhibition of fumarate oxidation, because the oxygen

uptake is high. With the other saturated acids (5 to 8 carbon atoms) the results are similar. Propionate, which does not increase the oxygen uptake and disappears at only about 20 per cent of the rate of butyrate, produces the same changes in phosphate as butyrate does. Decanoate and stearate, which decrease the oxygen uptake, also produced these changes.

The oxidation of fumarate appears to be necessary for obtaining the maximum speed of butyrate disappearance. The mechanism of this oxidation has been discussed by Lipmann (13). Succinate is oxidized by the system, but probably fumarate is not formed in sufficient amounts to produce an effect. Malate is less active than fumarate.

Our system does not oxidize higher fatty acids as does the enzyme preparation described by Lang *et al.*, although it contains adenylic acid and the method of preparation does not differ very much from theirs. However, the experimental conditions were slightly different because they measured the reduction time of methylene blue. Furthermore their enzyme is more stable and does not oxidize lower fatty acids; the addition of methylene blue to our enzyme did not enable it to oxidize higher fatty acids nor did it reactivate the enzyme after standing overnight in the ice box.

#### SUMMARY

The preparation of an enzyme system from liver which oxidizes lower saturated fatty acids is described. Inorganic phosphate, fumarate, cytochrome *c*, adenylic acid, and magnesium or manganese ions were found to be necessary components.

Fluoride, iodoacetate, arsenate, and malonate inhibit the oxidation. The changes occurring in phosphate distribution have been studied.

It is a pleasure for us to thank Dr. B. A. Houssay for his constant encouragement and interest in this work.

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# THE COLORIMETRIC DETERMINATION OF CHOLINE

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In the colorimetric determination of choline two methods are preferred: (a) determination as choline iodide (Roman (1)), (b) determination as the reineckate of choline (Beattie (2)).

Precipitation of choline by Reinecke salt, a method of adequate sensitivity, is perhaps the most widely employed method for the determination of this substance which can then be determined colorimetrically by means of the reddish color imparted to acetone by the reineckate of choline (Beattie). However, only rather concentrated solutions furnish sufficient color for accurate determinations by this method.

Rossi, Marenzi, and Lobo (3) studied photometrically the method for determining chromate ion ( $\text{CrO}_4^{2-}$ ), based on the reaction of Cazeneuve (4) which can be used for the determination of chromium in reineckates. This method has been used by us as the basis of a new method for the determination of choline, which is much more sensitive than those existing at present. We have also studied the use of Beattie's method for the estimation of choline.

## EXPERIMENTAL

*Preparation of Reineckate of Choline*—The reineckate of choline was prepared in a pure condition, according to the directions of Kapfhammer and Bisehoff (5). About 0.5 gm. of choline chloride was dissolved in 100 ml. of distilled water and 100 ml. of a saturated solution of ammonium reineckate were added, acidified with a few drops of HCl. The precipitation of the reineckate of choline is immediate, but the preparation is kept in the refrigerator for 12 hours in order to assure total precipitation. The precipitate is separated by centrifugation and washed twice with 96 per cent alcohol and once with ether. It is then dried *in vacuo* over sulfuric acid.

The choline reineckate is analyzed by determining its nitrogen by the Kjeldahl method and chromium as chromic anhydride ( $\text{Cr}_2\text{O}_3$ ) after ashing. The results obtained from nitrogen determinations were largely unsatisfactory, while the results obtained from ashing agreed with the theoretical values.

*Analysis of Choline Reineckate*— $\text{Cr}_2\text{O}_3$ . Calculated, 17.99; found, 18.2



In view of the unsatisfactory results derived from the nitrogen determinations, a purification of the salt and a new determination were undertaken.

To purify the choline reineckate, the salt was dissolved in the least quantity of acetone and filtered. The filtrate was diluted with a few drops of water, followed by shaking each time, until small brilliant crystals appeared. The solution was then kept in the refrigerator overnight to complete the precipitation. The crystals were centrifuged, washed once with 96 per cent alcohol and once with ether, and then dried *in vacuo* over sulfuric acid.

The nitrogen content of the choline reineckate was low, the same as the values obtained before purification, while the determination of chromium as the anhydride gave satisfactory results.

*Analysis of Recrystallized Choline Reineckate*— $\text{Cr}_2\text{O}_3$ . Calculated, 17.99; found, 18.35

The salt thus prepared was used in the experiments to be mentioned later.

#### *Photometric Study of Beattie Method*

The Pulfrich photometer was used in a study of the determination of choline according to the method of Beattie, with the choline reineckate which we had prepared.

The solubility of choline reineckate in aqueous acetone solutions of various concentrations was studied initially. Since acetone is a volatile solvent, it is inconvenient to use. It was found that 60 per cent acetone in water constituted a less volatile solvent which instantly dissolved choline reineckate. Lower strengths of acetone did not dissolve the salt completely, or else gave rise to a precipitate after some time.

*Standard Solution of Choline Reineckate*—34.86 mg. of the salt, equivalent to 10 mg. of choline, were dissolved in 60 per cent acetone; so that each ml. of this solution was equivalent to 100  $\gamma$  (0.1 mg.) of choline.

*Choice of Filter*—With this solution and a 30 mm. cube, the results of Table I were obtained. The low intensity of color in the solution renders necessary the use of a thicker cube in order that the readings may fall within the useful range of the photometer. The maximum absorption was noted with Filter S-53. Brante (6) advised the use of Filter S-50, which has a lower absorption, but did not indicate the basis of this preference nor details of the photometric observations.

*Calibration Curve*—Varying concentrations of choline reineckate were prepared in 10 ml. volumes of 60 per cent acetone. The readings were made with a 30 mm. cube and 60 per cent acetone. From these results

the specific extinction coefficient was calculated for 10 mm. thickness and for a concentration of 1 mg. of choline in a volume of 10 ml. (Table II). The specific extinction coefficient thus derived was 0.0921 and the calculation of concentration was made according to the formula

$$\frac{1}{0.0921} \times E_A = 10.85 \times E_A = \text{mg. choline in sample}$$

The results show that up to 500  $\gamma$  of choline can be determined on a volume of 10 ml. It is nevertheless possible to increase the sensitivity

TABLE I  
*Choice of Filter with Acetone Solutions of Choline Reineckate*

Filter No.	Maximal absorption, m $\mu$	$E_A$ = extinction, 30 mm. cube •
S-43	430	0.118
S-47	463	0.130
S-50	494	0.232
S-53	530	0.280
S-57	572	0.138
S-61	619	0.025
S-66	666	0.008
S-72	729	0.000
S-75	750	0.000

TABLE II  
*Determination of Specific Extinction Coefficient  $E_0$  of Solutions of Choline Reineckate*

Choline mg. per 10 ml.	$E_A$ , 30 mm. cube	$E_0$ = specific extinction coefficient, 10 mm. thickness, 1 mg. choline
1.0	0.278	0.0925
0.8	0.220	0.0915
0.6	0.165	0.0915
0.4	0.112	0.0931
Average.....		0.0921

of the reaction by using a smaller volume of solvent and then using micro cubes for the readings, but under these conditions it is difficult to determine concentrations below 100  $\gamma$  of choline. Therefore, we continued to study the new technique for the determination of choline, as described below.

#### *Determination of Choline by Estimation of Chromium in Choline Reineckate*

The proposed method is based on the oxidation of the chromium of choline reineckate to the chromic state by means of alkaline hydrogen

peroxide (perhydrol), followed by colorimetric determination of chromate by means of the color produced in acidic solution with a solution of diphenylcarbazide (Cazeneuve's reaction). The intensity of the color was measured with the Pulfrich photometer.

The technique of this determination is described below, but the preliminary experiments are given first.

*Reagents—*

Saturated solution of ammonium reineckate in distilled water, prepared at the time of using. The concentration of the solution is approximately 4 per cent.

96 per cent alcohol.

60 per cent acetone.

10 per cent NaOH.

Perhydrol.

10 per cent (by volume)  $\text{H}_2\text{SO}_4$ .

0.2 per cent diphenylcarbazide in 96 per cent alcohol. This solution at first has a faint rosy color, which becomes darker after a few days, but the solution can be used, nevertheless.

*Method—*The amount of choline to be determined varies between 15 and 100  $\gamma$  dissolved in water rendered slightly acid with dilute HCl. The volume of sample may range between 1 and 3 cm. The sample is placed in a centrifuge tube with a slender end ( $\pm 3$  mm.) and an equal volume of an aqueous saturated solution of ammonium reineckate. The tubes are then placed in ice water for at least 20 minutes to complete the precipitation of choline. Longer chilling does not affect the results.

The precipitate is separated by centrifugation for 4 minutes at 3000 R.P.M. Previous chilling of the copper tube carriers is helpful, as an increase in temperature favors the solution of the precipitate.

At the end of the period of centrifugation, the supernatant is separated by means of a small capillary tube with a fine end provided with a suction bulb. The liquid is removed as completely as possible without loss of the precipitate. The precipitate is then washed by running 0.5 ml. of 96 per cent alcohol, previously chilled in ice water, down the side of the centrifuge tube. The washing should be carried out with care lest some of the precipitate be lost. The alcohol is mixed with the residual liquid in the centrifuge tube by inclining the tube until it is almost horizontal and the tube is then stirred rapidly. This procedure is repeated two or three times, so that the excess of ammonium reineckate remaining after decantation is greatly lessened.

The tubes are chilled a few minutes and centrifuged as above. The washing is then repeated with 0.5 ml. of cold 96 per cent alcohol under the conditions mentioned above. The supernatant liquid after this second washing is almost colorless, but a third washing may be necessary.

The choline reineckate thus obtained is dissolved in about 1 ml. of acetone, warmed if necessary, and the solution is transferred to an ordinary test-tube. The centrifuge tube is then washed two or three times with 1 ml. of 60 per cent acetone and the washings are transferred to the same test-tube. The following are then added, 1 to 2 ml. of water, 0.2 ml. of sodium hydroxide, and 0.1 ml. of perhydrol for each 50  $\gamma$  of choline in the sample. An excess of perhydrol does not interfere but unnecessarily prolongs the period of heating in order to destroy the excess.

The test-tube thus prepared is placed in a boiling water bath. At first the heating must be conducted carefully, since the rapid evaporation of acetone may induce spattering. After most of the acetone is eliminated, the tubes are kept in the bath for 20 to 30 minutes, being shaken frequently in order to assist the decomposition of the perhydrol which must be completely destroyed, as otherwise no color will form. The final heating is conveniently carried out over a naked flame for a few seconds to insure the complete destruction of the perhydrol.

During the heating on the water bath the liquid acquires a yellow color from the formation of chromate; occasionally the initial color due to the chromate is deepened by a color produced by other substances, an indication of insufficient perhydrol. In this case, 0.1 ml. more of perhydrol is added and the heating is continued as described above.

After the oxidation of the chromium, the tubes are cooled, diluted with 3 to 4 ml. of water, 2 ml. of sulfuric acid are added, and sufficient diphenylcarbazide solution is added to yield a final concentration of 8 per cent. A violet-red color appears. The liquid is transferred to a suitable container and diluted to the appropriate volume.

The color was measured with the Pulfrich photometer equipped with Filter S-53 and a cube of convenient thickness. The observed extinction ( $E_h$ ) is converted to the standard volume of 1 cc. The comparison cube contains a blank consisting of 2 ml. of sulfuric acid and 2 ml. of diphenylcarbazide made up to 25 ml. with water.

*Calculations*—The specific extinction coefficient for 1  $\gamma$  of choline in a final volume of 25 ml. is 0.011; the calculation is therefore made by the following formula,

$$\frac{1}{0.011} \times E_h \times \frac{V}{25} = 90.9 \times E_h \times \frac{V}{25} = \text{micrograms choline in sample}$$

*Experiments*—The proposed method was finally reduced to a determination of chromium according to Cazeneuve's reaction, the best conditions for which were studied by Rossi, Marenzi, and Lobo who considered the selection of the filter, the effect of varying concentration of acid, diphenylcarbazide, and of possible interfering substances. It is therefore needless to repeat this work here.

*Determination of Specific Extinction Coefficient of Choline*—The quantity of chromate corresponding to choline was calculated from the formula of choline reineckate and a solution of barium chromate was prepared in dilute HCl containing 20.9 mg. per cent, which corresponds to 10 mg. per cent of choline; so that 1 ml. of solution contained 100  $\gamma$  (0.1 mg.) of choline. Dilutions of choline from 1 to 50  $\gamma$  were prepared from this solution, a volume of 25 ml. being used for quantities down to 10  $\gamma$  of choline and a volume of 10 ml. for smaller amounts. The results obtained

TABLE III  
*Determination of Specific Extinction Coefficient of Choline*

Choline	Volume of final dilution	$E_A$ per cm. thickness	$E_s$ per 1 gm. choline in 25 ml.
$\gamma$	ml.		
50	25	0.540	0.0108
40	25	0.415	0.0104
25	25	0.268	0.0107
20	25	0.210	0.0105
10	25	0.115	0.0115
5	10	0.143	0.0114
1	10	0.029	0.0116
Average . . . . .			0.011

TABLE IV  
*Determination of Choline in Choline Reineckate*

Choline calculated	Choline found	Difference
$\gamma$	$\gamma$	$\gamma$
50	48.17	-1.83
30	31.81	+1.81
25	24.54	-0.46
20	21.81	+1.81
10	10.91	+0.91

are shown in Table III. From these data it was calculated that the specific extinction coefficient for 1  $\gamma$  of choline in 25 ml. is 0.011. The calculation is carried out with the formula given above.

*Estimation of Choline Reineckate*—With choline reineckate made according to the method previously described, a 34.86 mg. per cent solution in 60 per cent acetone was prepared. Each ml. of this solution is equivalent to 100  $\gamma$  of choline. This solution was used for preparation of the dilutions mentioned in Table IV, which were placed in test-tubes and then subjected to the procedure previously described. The results were satisfactory.

Potassium persulfate and silver nitrate were tried as oxidizing agents for choline reinckate, but the results were unsatisfactory.

*Experiments on Precipitation of Choline*—A solution of choline was determined by the method of Beattie and various dilutions of it were prepared as shown in Table V.

At first the effect of temperature on the precipitation of choline reinckate was studied, and it was shown that the precipitation was quantitative in the cold, was complete in 20 minutes in an ice water bath, and that a longer period in the ice water did not influence the results. The precipitation should be carried out in neutral or slightly acid media.

The effect of variations in the volume of liquid before precipitation was also studied. Satisfactory results were obtained when the choline to be

TABLE V  
*Experiments on Precipitation of Choline*

Choline calculated	$E_A$ found	Final volume	Choline found	Difference	Error
$\gamma$		ml.	$\gamma$	$\gamma$	per cent
66.94	0.730	25	66.35	-0.59	-0.88
66.94	0.700	25	63.63	-3.31	-4.94
66.94	0.760	25	69.08	+2.14	+3.19
40.20	0.440	25	39.99	-0.21	-0.52
33.47	0.380	25	34.54	+1.07	+3.20
33.47	0.390	25	35.45	+1.98	+5.92
33.47	0.360	25	32.72	-0.75	-2.24
33.47	0.372	25	33.81	+0.34	+1.02
33.47	0.366	25	33.27	-0.20	-0.60
20.10	0.240	25	21.81	+1.71	+8.51
16.73	0.420	10	15.27	-1.46	-8.73
16.73	0.180	25	16.36	-0.37	-2.21
16.73	0.172	25	15.63	-1.10	-6.57

precipitated is contained in from 1 to 3 ml. and an equal volume of an aqueous saturated solution of Reinecke salt is added.

Numerous preliminary experiments demonstrated that choline reinckate is rather soluble in the liquids used for washing the precipitate. This led us, in the first place, to decrease the volume of wash water, and, in the second place, to carry out the washings without removing the precipitate. The most appropriate washing fluid is 96 per cent alcohol chilled in ice water. The washing is carried out with two 0.5 ml. portions of cold alcohol, decanted each time with the aid of a capillary tube drawn out at the end attached to a suction bulb, without removal of the precipitate. During the washing, great care is necessary to avoid loss of the precipitate through solution. Decantation must always be done carefully,

since the residual liquid which contains Reinecke salt is a source of high values. When these precautions are followed strictly, the recovery of choline from solutions of known concentration is satisfactory, as indicated by the results in Table V.

If the solubility of choline reineckate is taken into consideration, together with the small amount of material which is precipitated, the results obtained will be seen to be perfectly acceptable, even though the per cent error may appear excessive. Amounts of choline below 15  $\gamma$  cannot be determined, since the error derived from the solution of the precipitate becomes too great.

#### SUMMARY

The method for the determination of choline proposed by Beattie was studied photometrically.

A new method for the determination of choline was established, based on the precipitation of choline as the reineckate and colorimetric determination of the chromium in the precipitate, according to the method of Cazeneuve.

Under the conditions defined by us, this method allows choline to be determined in samples containing as little as 15  $\gamma$ .

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# ON THE DETERMINATION OF THE PHOSPHOLIPIDS IN BLOOD

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Our previous investigations on the determination of choline led us to study the application of the method to the determination of the phospholipids of the blood and to their distribution as lecithin, cephalin, and sphingomyelin.

The early studies in this direction were devoted to determinations of some of these individual constituents. In 1929, Bloor (1) proposed the separation of the monoamino monophospholipids (lecithin and cephalin) by precipitation from acetone with magnesium salts, followed by oxidation with dichromate-sulfuric acid. Kirk, Page, and Van Slyke (2) in 1934 estimated cephalin by determination of the amino nitrogen in petroleum ether solutions of extracts of plasma or tissues. Folch and Van Slyke (3) showed, however, that petroleum ether in the presence of phosphatides dissolves significant amounts of urea and amino acids from the residues of the alcohol-ether solutions, and that the redissolved urea and amino acids cause cephalin values estimated from amino nitrogen to be much too high.

In 1936, Thannhauser and Setz (4) described a method for the estimation of sphingomyelin by precipitation with Reinecke salt, for which modifications were later proposed. In 1938, Williams, Erickson, Avrin, Bernstein, and Macy (5), making use of the method for choline described by Beattie (6), suggested a method for lecithin and cephalin, based on the determination of the choline-P ratio of the monoamino monophospholipids precipitated with  $MgCl_2$  according to Bloor. Thannhauser, Benotti, and Reinstein (7) in 1939 published a method for lipid P, choline phospholipids, and sphingolipids, from which cephalin was calculated indirectly. Some refinements of this technique were introduced by Erickson, Avrin, Teague, and Williams (8) in 1940, who also determined cephalin by difference.

Brante (9) in 1940, making use of a similar method, calculated cephalin indirectly from the ratio between choline phospholipid and total phospholipid. Blix (10) in 1940, on the other hand, determined total phospholipid, fatty acids, and glycerol, from which the sphingomyelin concentration could be calculated indirectly.

Our investigations were based on those of Thannhauser, Benotti, and Reinstein. Total phospholipid was estimated by determination of lipid P,



sphingomyelin by precipitation with Reinecke salt and determination of the phosphorus in the precipitate, and choline phospholipids after hydrolysis. The three phospholipids were determined from these values, cephalin being determined by difference. It was found necessary to study separately each step in the fractionation, and therefore each step in the method used is described in the following.

#### EXPERIMENTAL

*Extraction of Total Lipid*—For extraction of plasma lipids, Bloor used a mixture of 3 parts of 98 per cent ethanol and 1 part of ether and reextracted with petroleum ether. The dilution of plasma was 1 to 19 parts of the alcohol-ether mixture. The same technique has been used by many workers. Thannhauser and Setz extracted the lipids from plasma or desiccated plasma with a mixture of equal parts of chloroform or methanol. This method has the advantage of avoiding the presence of water and a smaller amount of solvent is used.

A number of workers are not in agreement as to the advantages of the reextraction of the extract with petroleum ether according to Bloor. While Kirk and collaborators (2) believe that reextraction with petroleum ether gives good results, Man (11), Ellis and Maynard (12), Williams and collaborators (5), and Erickson and coworkers (8) maintain that petroleum ether does not completely extract phospholipids.

We have compared extraction of plasma phospholipids according to Bloor without reextraction with petroleum ether and with extraction of plasma dried on filter paper by the same solvent.

The following procedure was followed for extraction of lipids. An absorbent, fat- and P-free paper absorbs 2.5 ml. of plasma and is dried at 50°. Drying is complete in 10 to 20 minutes. The paper is cut into small pieces and placed in a 100 ml. flask. There are added 25 to 30 ml. of a mixture of 3 parts of ethanol and 1 part of ether, so that the paper is completely covered. The flask is then fitted with a condenser and refluxed for 20 minutes on a water bath. The flask is allowed to cool a bit, and the liquid decanted into a 50 ml. test-tube. The paper left in the flask is washed four to five times with 5 ml. portions of alcohol-ether, the flask being heated slightly, and the washings are added to the test-tube, so that the final volume of cooled extract is 50 ml. In this manner, a plasma dilution is obtained similar to that of Bloor (1:20).

Extraction by this method was compared with the Bloor method by determination of total lipid P in the extract. Table I shows the results. Very similar results are obtained with both methods. Except in two instances, the Bloor method gives somewhat higher values, which are attributed to the error arising from the volume of the precipitate. Drying

on paper allows the plasma to be kept for several days in good condition, as we have had opportunity to note.

**Determination of Total Lipoid P**—This determination is carried out on 10 ml. of the alcohol-ether extract, which is equivalent to 0.5 ml. of plasma, according to the technique of Fiske and Subbarow (13). If the determination is carried out with the Pulfrich photometer with Filter S-75 and a 10 mm. thickness, the calculation is performed according to the following equation.

$$0.143 \times E_A \times \frac{100}{0.5} = 28.6 \times E_A = \text{mg. \% lipid P}$$

$$\text{Lipoid P} \times 25.5 = \text{mg. \% phospholipid}$$

**Determination of Sphingomyelin**—The determination of this substance is based on its precipitability by Reinecke salt from methanol.

TABLE I  
Comparison of Methods for Extraction of Lipids

Plasma	Method of Bloor		Drying on paper	
	Lipoid P	Phospholipid	Lipoid P	Phospholipid
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Dog.....	12.44	317	13.38	341
" .....	14.01	357	13.79	351
" .....	8.44	215	8.15	207
Human.....	7.01	178	6.58	167
" .....	7.87	200	8.01	204
" .....	5.92	150	5.86	149
" .....	6.95	177	6.86	174

In theory the determination may be carried out in two ways: by determination of P or by measuring the color of the precipitate.

Colorimetry of the precipitate did not yield satisfactory results. An alcohol-ether extract is prepared from 250 ml. of plasma. The extract is dried in a vacuum and extracted with chloroform to eliminate any free choline. The chloroform extract is then dried and taken up in methanol. An equal volume of saturated ammonium reineckate in methanol is then added and kept in the refrigerator overnight.

The precipitate is centrifuged and washed with methanol, acetone, and ether. The ether dissolves enough of the precipitate to acquire a gray-green color. The washed precipitate is dried *in vacuo* over sulfuric acid.

The phosphorus, chromium, and choline content of the reineckate of sphingomyelin thus obtained is determined in duplicate, with the results shown in Table II, in which the theoretical values for the reineckate of sphingomyelin are given according to Thannhauser and coworkers.

The results indicate that phosphorus is the only component whose concentration approaches the theoretical value.

The precipitate contains a greater proportion of choline than of phosphorus, an indication that the extract, in spite of reextraction with chloroform and washing of the precipitate with acetone, contains free choline.

On the other hand, the precipitate has less choline than chromium, which would indicate that Reinecke salt precipitates something that is not choline, since the residual chromium ( $8.22 - 4.59 = 3.63$  per cent chromium) is almost equal to the residual choline ( $14.60 - 10.68 = 3.92$  per cent choline). If all the excess choline were bound as reineckate, 1.56 per cent of chromium would be fixed and 2.36 per cent of chromium would be left over as the unknown compound.

It was concluded from this study of the precipitation of sphingomyelin as the reineckate that under ordinary working conditions it is not possible

TABLE II  
*Analysis of Reineckate of Sphingomyelin*

	Calculated	Found
	<i>per cent</i>	<i>per cent</i>
Phosphorus.....	2.73	2.86
Chromium.....	4.59	8.22
Choline.....	10.68	14.60
	<i>ratio</i>	<i>ratio</i>
Choline-P.....	3.89	5.10
Choline-chromium.....	2.33	1.78
Chromium-P.....	1.68	2.87

to determine sphingomyelin by determining the chromium in the precipitate, and that only the value for P is correct.

In addition, numerous simultaneous determinations of chromium and phosphorus were carried out on the precipitates derived from the plasma extracts, but no constant ratio between both these components was found. Their ratio varied from 1.70 to 2.90, indicating that the precipitate was not constant in composition, a logical conclusion from the results detailed above.

We then hoped to determine sphingomyelin by determining phosphorus in the reineckate of sphingomyelin, although this would require the use of a greater amount of plasma. The method worked out is as follows: Place 30 ml. of alcohol-ether extract in a 150 ml. beaker (equivalent to 1.5 ml. of plasma) and evaporate carefully on the water bath, shaking and avoiding overheating of the extract. The dry residue is taken up in methanol. 2 ml. of methanol are added and carefully brought to a boil, half of the

liquid allowed to evaporate, and the remainder transferred to a 15 ml. centrifuge tube. The beaker is washed, with the same precautions, with three 1 ml. portions of methanol, and the washings transferred to the centrifuge tube, which should contain 2 to 3 ml. of a turbid solution of phospholipids in methanol. 2 to 3 volumes of a saturated solution of ammonium reineckate in methanol, acidified with a few drops of hydrochloric acid and filtered at the time of using, are then added. The tube is stoppered and placed in an ice-water mixture for at least 1 hour. A longer time does not affect the results.

Sometimes an abundant precipitate forms, and at other times a fine precipitate which settles easily.

The precipitate is then centrifuged for 4 to 5 minutes at 3000 R.P.M. The supernatant is decanted by inversion of the tube, and the mouth of the tube is cleaned with filter paper. The residue is first washed with 2 ml. of cold methanol, the precipitate being suspended in the liquid with the aid of a slender glass rod. The tube is restoppered and again chilled for 5 minutes, and centrifuged as before. The supernatant is decanted and the residue washed with 2 ml. of acetone, the precipitate being suspended in the acetone as indicated for methanol. The tube is then cooled in ice water for 5 minutes, centrifuged, and decanted as before.

The residue is then dissolved at room temperature in 1 ml. of 5 N  $H_2SO_4$  and is transferred to a large combustion tube to ash the phosphorus, according to the technique of Fiske and Suhharow. The centrifuge tube is washed twice with methanol-acetone to assure a quantitative transfer. The remainder of the procedure is the same as that followed in the determination of total lipid P. At the end of the combustion the residue is transferred to a 10 ml. tube with the aid of several portions of water until a volume of 7 to 8 ml. is reached. As the solution thus obtained is always slightly turbid, it is convenient to filter it through a small fine filter, which is then washed.

To the solution thus obtained are added 1 ml. of molybdate and 0.4 ml. of the reducing reagent. Water is added to bring the volume up to 10 ml. and the color is measured in a photometer 5 minutes after the reaction by use of Filter S-75 in a depth of 10 mm.

The calculation of sphingomyelin is carried out according to the following equation.

$$E \times 0.0715 \times \frac{100}{1.5} = E \times 4.767 = \text{mg. \% P}$$
$$\% P \times 26.84 = \text{mg. \% sphingomyelin}$$

Various experiments were performed before the technique described above was established, having to do for the most part with the washing

of the precipitate, to determine whether it was possible to obtain a product with a constant chromium-phosphorus ratio.

It was also observed that an excessive number of washings, as advised by Erickson and collaborators, always leads to an appreciable loss of P. Also, ether cannot be used as the wash liquid for the reineckate of sphingomyelin because of the appreciable solubility of the compound in ether.

Previous extraction of the alcohol-ether extract with chloroform, in order to eliminate choline, is of no advantage in the determination of sphingomyelin, since chromium cannot be determined for the reasons previously described and would represent merely a complication of the method.

*Determination of Choline Phospholipids (Lecithin and Sphingomyelin)*—In the determination of choline by the chromium content of the reineckate of choline, obtained after the hydrolysis of the phospholipids, it is possible to diminish considerably the amount of alcohol-ether extract required. The choline phospholipids can be determined on a 0.25 to 0.50 ml. sample of serum (equivalent to 5 to 10 ml. of alcohol-ether extract).

The method in use by us is the following. Place 5 ml. of alcohol-ether extract in a 100 ml. beaker and evaporate on the water bath, avoiding overheating of the residue. The residue is reextracted two to three times with small portions of chloroform (2 ml. per portion) in the cold, in order to eliminate free choline as much as possible. The chloroform extract is then transferred to a small round bottomed flask and the chloroform is evaporated on the water bath. There are added to the residue 2 ml. of 5 N HCl in methanol, according to the method of Thannhauser, Benotti, and Reinstein, who used a reflux condenser and heated the extract with a small flame for 3 hours in order to hydrolyze the phospholipids.

At the end of the heating, the methanol-HCl is evaporated on the water bath to a small volume and the final drying is conducted *in vacuo*. The residue is taken up in small portions (0.5 ml.) of 2 per cent aqueous HCl; five to six of these washings are transferred to a 15 ml. centrifuge with a narrow tapered end. It is convenient to filter the extraction liquid through a small moistened cotton filter in order to retain the fatty acids liberated by hydrolysis. The liquid thus obtained is precipitated with an equal volume of saturated aqueous ammonium reineckate, and the remainder of the procedure follows the lines laid down for the determination of choline (Marenzi and Cardini (14)).

When the extract is made according to Bloor's directions, at times a copious precipitate forms and it is then convenient to carry out the first washing, the precipitate being stirred up with a slender glass rod.

By the technique described above, the development of the final color can be carried out on a volume of 25 ml. and the calculation is then performed according to the following formulae.

$$90.9 \times E \times \frac{100}{0.25} = 36.360 \times E = \text{micrograms \% choline}$$

or

$$36.36 \times E = \text{mg. \% choline}$$

$$\text{Choline} \times 6.7 = \text{choline phospholipid}$$

We have carried out the hydrolysis with NaOH according to Brante with good results. The method adopted was the following. 5 ml. of the alcohol-ether extract are taken up in chloroform and transferred to a 50 ml. flask. 5 ml. of absolute alcohol and 0.5 ml. of 2 N NaOH are added, and the flask heated on a water bath under a reflux for 1 hour. The extract is then transferred to a beaker and evaporated. When only 1 ml. remains, the alkali is neutralized to phenolphthalein with 2 N HCl. The evaporation is carried to dryness and the procedure then is the same as for the previous hydrolysis with methanol-HCl.

TABLE III  
*Distribution of Phospholipids in Human Plasma*

	Total phospholipid	Choline phospholipid	Lecithin	Cephalin	Sphingomyelin	Choline
Mg. %.....	203.74	159.67	126.78	42.71	35.35	23.83
% total phospholipid.....		78.49	61.46	21.50	17.05	

Barium hydroxide hydrolysis was not tried, since, according to Thannhauser, no more than 60 per cent of the sphingomyelin is hydrolyzed and the method is complicated by the necessity of eliminating the barium.

*Distribution of Phospholipids in Plasma*—The following relations were calculated: total phospholipid minus choline phospholipid = cephalin; choline phospholipid minus sphingomyelin = lecithin; sphingomyelin determined directly.

The results obtained with human plasma are shown in Table III. These results differ in general from those of Thannhauser and Erickson. The per cent of lecithin obtained by our method is greater than that obtained by Erickson. The per cent of cephalin differs notably from that obtained by Erickson and by Thannhauser, but approximates the value obtained by Brante (22 per cent). The per cent of sphingomyelin is very close to that obtained by Erickson and is greater than that assigned to it by Thannhauser and by Blix (13 per cent).

#### SUMMARY

The method for the determination of choline described by the writers was applied to the determination of the choline phospholipids of plasma.

A study of the composition of the reineckate of sphingomyelin demonstrated that the pure salt is not precipitated from alcohol-ether extracts of plasma and that sphingomyelin can be determined only from the phosphorus content of the precipitate.

Values were established for human plasma and the percentage of distribution of the three phospholipids.

The conversion factors from phosphorus and choline to phospholipid received a theoretical treatment.

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## THE METABOLISM OF ESTRONE IN MEN\*

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In 1938, Dingemanse *et al.* (1) isolated estrone from the urine of men; one-half to two-thirds of the total estrogenic activity was found in the non-ketonic fraction. In an endocrine-cancer study conducted in this laboratory (2), information was obtained regarding the nature of the non-ketonic estrogens normally excreted by men; these appeared to be estradiol and estriol. More recently, an increase in the estrogenic titers of certain urine fractions was observed after the administration of estrone to non-cancerous men (3); the physical and chemical properties of the substances responsible for the estrogenic activity of the non-ketonic "weak" phenols and the "strong" phenols resembled closely those of estradiol and estriol. More direct evidence was considered desirable. Large quantities of estrone were injected into men and an attempt was made to isolate the estrogenically active metabolites of the ketonic hormone. The outcome of this investigation is now reported.

### EXPERIMENTAL

*Administration of Estrogen*—A total of 1.05 gm. of estrone acetate, m.p. 124–125°<sup>1</sup> (prepared from estrone which had been obtained from pregnant mare urine), was dissolved in olive oil and injected intramuscularly into seven healthy young men. No more than 50 to 160 mg. of estrone acetate was injected at any one time and no man received more than 160 mg. in all. Two of the subjects experienced nausea and vomited within 24 hours following the injection. Hypertrophy of the breast and tenderness of the nipples became evident in several subjects within a week; regression extended over a period of a few weeks.

*Collection, Hydrolysis, and Extraction of Urine*—The urine was collected for 96 hours following estrogen administration; 30.7 liters were obtained. The urine was preserved with toluene and hydrolyzed within 48 hours after collection by boiling with 15 per cent concentrated HCl (by volume) for 7 minutes. The hydrolysate was rapidly cooled and extracted with ethyl ether.

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<sup>1</sup> All melting point values reported are corrected.



*Fractionation of Urine Extracts*<sup>2</sup>—The ether-soluble material of the urine was separated into acids, phenols, and neutral substances by usual methods. The phenols were taken up in benzene and extracted with 0.3 M  $\text{Na}_2\text{CO}_3$  in order to obtain the estriol fraction and the procedure then repeated. A sharp separation of estriol from other estrogens is thereby effected. Mather (5) had previously determined the partition ratios for crystalline estriol and other estrogens. His findings have been confirmed by Bachman and Pettit (6) and also by us (3). Mather also found that the partition ratios were essentially the same when crystalline estrogens were added to crude urinary extracts (private communication). The distributions between benzene and sodium carbonate have been used to advantage by other investigators (6-9) and also previously by us (3) in working up crude estrogen extracts.

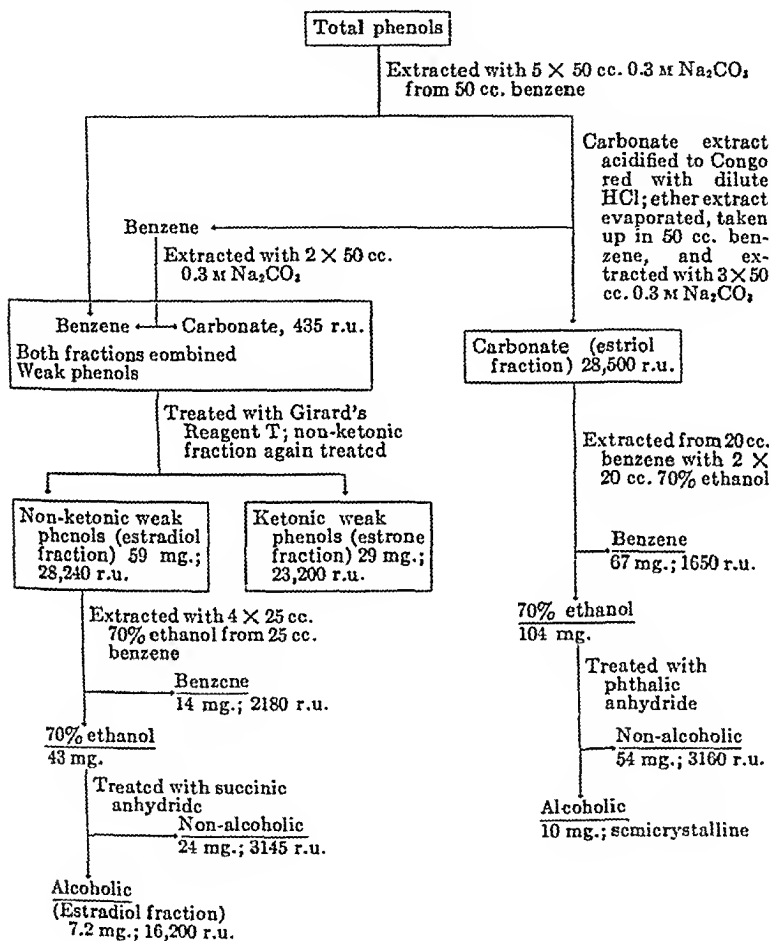
The weakly acidic phenols (benzene phase) were fractionated into ketones and non-ketones by the method of Girard and Sandulesco (10) with the use of Reagent T. The non-ketonic "weak" phenols (estradiol fraction) and the "strong" phenols (estriol fraction) were treated with succinic and phthalic anhydride respectively under conditions previously described (11). The hydrolysis of the succinic acid esters was carried out at room temperature for 24 hours with 5 per cent KOH in 90 per cent methanol; the period of hydrolysis was 48 hours in the case of the phthalic acid esters. The hydrolysates were poured into water, acidified to Congo red with dilute HCl, and extracted with ether. The anhydrides of succinic and phthalic acids have been extensively employed in removing hydroxylated steroids from non-hydroxylated substances. Although estrone possesses a hydroxyl group, it is not esterified by the aforementioned procedures; however, hydroxyl groups in the non-benzenoid portion of the estrogen molecule, as in  $\alpha$ -estradiol or estriol, will react (3). The quantitative separation of micro amounts of estrogens into "alcohols" and "non-alcohols" with the aid of succinic anhydride has been demonstrated (3).

When phthalic anhydride was permitted to react with 350  $\gamma$  of estriol, 78.4 per cent of the original estrogenic activity was recovered in the "alcoholic" fraction and 3 per cent in the "non-alcoholic." An estimate may thereby be obtained of the recovery of estriol after the urine fraction has been treated with phthalic anhydride. The esterification procedure serves as an effective check on the efficacy of the ketone separation. It also results in considerable enrichment of the non-ketonic fraction of the urine extract in estrogenic activity (*cf.* flow sheet). The dicarboxylic anhydrides have found commercial application in the extraction and purification of the estrogenic hormones of pregnancy urine (12).

<sup>2</sup> A preliminary report has appeared (4).

The course of the fractionation of the urinary estrogens is described in the flow sheet; the weights of the various fractions and their estrogenic

*Fractionation of Urinary Phenols*



activities are indicated. Bioassay was performed on spayed adult rats by a method previously described (2), with the vaginal smear technique. The estriol fraction finally gave 10 mg. of a semicrystalline product which

yielded from ethyl acetate 1.6 mg. of crystals, m.p. 266-268°. Recrystallization from the same solvent gave 0.6 mg., m.p. 269-270°; a mixed melting point determination with estriol (see Table I), m.p. 271-273°, was 268-272°. An additional 2 mg., m.p. 268-270°, were obtained from the mother liquors; the acetate after a single crystallization from aqueous methanol melted at 115-118°, gave no melting point depression with estriol acetate, m.p. 124.5-125.5°, but caused a marked depression in melting point on admixture with estrone acetate, m.p. 124-125°. A comparison of the properties of the estriol isolated and estriol is presented in Table I; a close similarity is apparent. It is very unlikely that the estriol isolated arose from endogenous estrogen and not from the estrone injected, in view of the extremely low estriol (2, 3) and total estrogen (1-3) content of the urine of males. Furthermore, when 0.051 gm. of estrone acetate was administered to one of the authors (W. H. P.) in a preliminary run, the estriol titers of the urine for the first, second, and third 48 hours were 825, 342, and 38 rat units respectively. Incidentally, it was for this reason that the urine collections were made for the first 96 hours following estrogen injection.

The non-ketonic "weak" phenols (estradiol fraction) finally yielded 7.2 mg. of brown oil which assayed for 16,200 rat units of estrogenic activity or an equivalent of 2.03 mg. of  $\alpha$ -estradiol. An attempt to obtain the crystalline estrogen with the use of digitonin (17) was unsuccessful when the method of Huffman *et al.* (8) was employed; the digitonin precipitation was carried out at approximately 5° instead of at room temperature. The ketonic "weak" phenols (estrone fraction) yielded on repeated recrystallization from methanol 1 mg. of impure estrone, m.p. 250-253°, which gave no depression in melting point on admixture with authentic estrone, m.p. 258-259°.

*Oxidation of Estradiol Fraction*—There is reason to believe that the estrogen components of the "estradiol" fraction are either  $\alpha$ - or  $\beta$ -estradiol or both. The crystalline steroids and the estrogenic material contained in the fraction are non-ketonic, "alcoholic," and weakly acidic phenols. We have previously shown (3) that the estrogenic activity of a similar "estradiol" fraction was partitioned between benzene and 70 per cent ethanol in a manner identical with that of crystalline  $\alpha$ -estradiol. Stereoisomers of estradiol have been isolated from pregnancy urine (8, 18, 19) and after the administration of estrogen to non-pregnant animals (20-22). Estrogens of the equilin and equilenin series appear to be confined to the Equidae.

Assuming that both are present, it was calculated that the 7.2 mg. of oil (16,200 rat units) obtained from the "estradiol" fraction contained 1.97 to 2.03 mg. of  $\alpha$ -estradiol and a maximum of 5.2 mg. of  $\beta$ -estradiol. The

presence of  $\alpha$ -estradiol can be detected and the relative amounts of the  $\alpha$  and  $\beta$  isomers calculated by indirect methods involving oxidative procedures. Thus, if  $\alpha$ -estradiol is quantitatively oxidized to estrone, the estrogenic activity would be only one-eighth of that originally present, since this is the relative order of activity of these steroids by our method of assay. With  $\beta$ -estradiol, an increase of 12.5 times in the activity should occur. If a mixture of isomeric diols were oxidized, and the conversion factor determined, the quantity of each component originally present is calculable. The wide divergence in the relative activities of

TABLE I  
*Comparison of Certain Properties of Estriol Isolated and Estriol*

Property	Estriol isolated	Estriol
M.p., °C.....	269-270	271-273*
Estrogenic potency (1 $\gamma$ ), rat units.....	1.05	1.0
% total activity extracted from benzene by 0.3 M Na <sub>2</sub> CO <sub>3</sub> †..	98.9	98.0
% " " " " " 70% ethanol†..	82.4	86.8
Absorption maxima in David (15, 16) color test, $m\mu$ .....	655, 510	655, 510

\* The melting point of the estriol used for purposes of comparison is 10° lower than that reported for pure estriol (13). It has been suggested that contamination with estrone might account for the low melting point values reported in the literature for this steroid (14). However, our specimen of estriol is almost completely extracted from benzene by sodium carbonate, whereas little or no estrone is similarly extracted (5). Furthermore, when the estriol was treated with phthalic anhydride, of the total activity recovered, 96 per cent went into the "alcoholic" fraction; estrone behaves as a "non-alcoholic" substance under similar treatment. A mixture of estriol, m.p. 271-273° corrected, and authentic estrone, m.p. 258.5-259.5° corrected, melted at 242-255° corrected. Contamination, not with estrone but possibly with some non-ketonic substance, may account for the low melting point of the estriol which was at our disposal.

† Two extractions with equal volumes of both solvents.

‡ One extraction with equal volumes of solvents.

$\alpha$ -estradiol, estrone, and  $\beta$ -estradiol would permit a fair degree of precision in such calculations. In our studies the mild oxidative procedure of Oppenauer (23) was employed.

Practically no destruction of steroid material resulted when 167 rat units of estrone were subjected to the Oppenauer oxidation; 93 per cent of the estrogenic activity was recovered in the ketonic fraction after treatment with 0.5 gm. of Girard's Reagent T. The oxidation of estradiol was, however, not complete, judging from the activity remaining in the non-ketonic fractions. This was taken into account in calculating conversion factors for  $\alpha$ - and  $\beta$ -estradiol, respectively. The data for the oxidation of the crystalline isomeric estradiols and of the urine concentrate

are given in Table II; the method of calculating the factors is indicated. As can be seen from Table II, the observed and theoretical conversion factors for the crystalline estrogens agree fairly well. Since  $\alpha$ -estradiol is more rapidly oxidized (approximately 1.4 times) in the Oppenauer reaction than is  $\beta$ -estradiol, the conversion factor cannot be strictly applied in the analysis of mixtures of the two isomers. None the less, it is obvious

TABLE II  
Oppenauer Oxidation of Estrogens

Substance oxidized	Estrogenic activity			Conversion factor*
	Before oxidation	After oxidation		
		Non-ketones	Ketones	
	<i>rat units</i>	<i>rat units</i>	<i>rat units</i>	
$\alpha$ -Estradiol	1815	370	173	0.119
	1815	478	131	0.099
Average found . . . . .				0.11
Theoretical†. . . . .				0.125
$\beta$ -Estradiol	17.9	8	113	11.9
	130	91.4	732	19.4
Average found . . . . .				16
Theoretical† . . . . .				12.5
Urinary estradiol fraction†	1144	617	62	0.118
	1144	299	61.5	0.070
Average found. . . . .				0.094

\* Conversion factor =

ketonic rat units (after oxidation)

rat units (before oxidation) minus non-ketonic (after oxidation) rat units.

† Theoretical values were obtained on the assumption that estrone is the oxidation product. The estrogenic activities for 1  $\gamma$  of crystalline  $\alpha$ -estradiol,  $\beta$ -estradiol, and estrone are 8.0, 0.08, and 1.0 rat units respectively.

‡ Most of the estrogenic activity was recovered after digitonin treatment of the 7.2 mg. of oil obtained from this fraction; aliquot portions were oxidized. The period of refluxing in the oxidations was 6 to 12 hours. The ketones were treated once more with the Girard's reagent in all cases except in the oxidation of the  $\alpha$ -estradiol.

that little or no  $\beta$ -estradiol can be present in the urine fraction, since the conversion factor obtained for this fraction so closely approaches that for pure  $\alpha$ -estradiol. The oxidative procedure employed was as follows: The desiccated steroid preparation was dissolved in 5 cc. of dry acetone to which was added 0.5 gm. of aluminum *tert*-butoxide dissolved in 10 cc. of dry benzene. The solution was refluxed for 6 to 12 hours, permitted to cool, and added to 20 cc. of ether. The ether was washed with dilute

HCl, then with water, and evaporated. The residue thus obtained was resolved into ketonic and non-ketonic moieties (10) and submitted to bioassay.

#### DISCUSSION

Our data clearly indicate that men can effect a partial conversion of administered estrone to estriol. Other investigators have shown that this process occurs in the mammalian organism but the evidence submitted was of indirect nature. Thus, Smith and Smith (24) showed that excessive amounts of estriol were excreted in the urine of women after the administration of estrone. Pincus and Pearlman (3) confirmed these findings and secured additional indirect evidence that the estrogen metabolite in question was indeed estriol. Pincus and Zahl (25) found that the presence of a functional uterus in the rabbit was essential to the estrone-estriol conversion. It appears from our present findings that the uterus is not essential, at least not in the human species. It is possible, however, that the uterus normally facilitates this transformation. Longwell and McKee (9) injected dogs with estrone and obtained from the bile a carbonate fraction possessing estrogenic activity. It is quite likely that estriol is the estrogen responsible for the activity of this fraction in view of the fact that estriol alone,<sup>3</sup> of the various naturally occurring estrogens, is extractable from benzene by a sodium carbonate solution.

A conversion of  $\alpha$ -estradiol to estriol in the animal body is theoretically possible in view of the fact that  $\alpha$ -estradiol is partially converted to estrone *in vivo* (26, 27), and we have shown that the latter steroid may in turn give rise to estriol. Doisy, Thayer, and Van Bruggen (28) found evidence for the excretion of estriol in an ovariectomized-hysterectomized monkey after the injection of  $\beta$ -estradiol. It is not, however, established that the isomeric estradiols must first be transformed to estrone and the latter in turn to estriol. Fieser (29) and also Marrian (30) suggested an intermediate step involving the hydration of the enolic form of estrone.

Oxidation of estriol may lead to the formation of 16-ketoestrone, according to a theory proposed by Marrian (30). This steroid has been recently prepared from estrone by Huffman (31); a 16-hydroxyestrone and a compound isomeric with estriol were also obtained. A study of the properties of these compounds may be useful in the search for estriol metabolites. One might expect, however, that a 16,17-diketone would be very susceptible to oxidation in the organism, leading to the formation

<sup>3</sup> It was considered of interest to determine the partition coefficient of  $\beta$ -17-dihydroequilenin, an estrogen possessing rather strongly acidic properties (19). A specimen kindly furnished by Professor O. Wintersteiner, when partitioned between equal volumes of benzene and 0.3 M Na<sub>2</sub>CO<sub>3</sub>, remained chiefly in the organic phase; 92.5 per cent of the total estrogenic activity recovered was found therein.

of the corresponding dicarboxylic acid. Alkaline fusion of estriol will lead to the formation of a degradation product of the latter type (32, 13). The glycol group of estriol is readily oxidized by lead tetracetate; a 16,17-dialdehyde is probably formed (8). A search will be made for oxidation products of estriol in the acid fraction of the urine extracts obtained in our study.

The fact that estriol is not converted to any appreciable extent to estrone or estradiol, whereas the converse is true, leads one to expect estriol to be the chief estrogen metabolite excreted. This appears to be the case in human pregnancy. However, estriol has not been isolated from the urine of species other than the human, although there is some evidence for the normal occurrence of estriol in the urine of non-pregnant chimpanzees (33). That estriol is no less resistant to destruction than are the other estrogens has been demonstrated (28); the inactivation process may be that already alluded to and may also include other mechanisms for the inactivation of estrogens, as recently reviewed by Heard and Hoffman (27).

Theoretically, both  $\alpha$ - and  $\beta$ -estradiol may arise as the result of the *in vivo* reduction of estrone. In the rabbit, only  $\beta$ -estradiol has been recovered after the administration of estrone (20); when  $\alpha$ -estradiol is given, fairly large amounts of  $\beta$ -estradiol are recovered from the urine (21, 22). Our data indicate that in men the formation of the  $\alpha$  isomer of estradiol is favored after the administration of estrone. Heard and Hoffman (27) recovered no  $\beta$ -estradiol after the injection of  $\alpha$ -estradiol into a young man. It is interesting that  $\alpha$ -estradiol, but as yet no  $\beta$ -estradiol, has been isolated from human pregnancy urine (8), whereas the urine of pregnant mares has yielded both diols (18, 19).

The assistance of Miss Mary Ruth Jones is gratefully acknowledged. Dr. Oliver Kamm of Parke, Davis and Company kindly supplied the estriol. Dr. Erwin Schwenk of the Schering Corporation donated the  $\alpha$ -estradiol. We are especially indebted to Dr. R. D. H. Heard for the  $\beta$ -estradiol.

#### SUMMARY

Massive doses of estrone, as the acetate, were injected into seven young men. From the strong phenolic fraction of the urine which was collected for 96 hours following the injection, there was recovered a small amount of crystalline estriol. An exogenous origin of the estriol isolated is precluded.

The high concentration of estrogenic activity in the weakly acidic, phenolic, non-ketonic fraction appears to be due chiefly to  $\alpha$ -estradiol. This is substantiated by the changes in the activity of this fraction after the application of mild oxidative procedures.

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# THE CONVERSION OF TRYPTOPHANE TO A PLANT GROWTH SUBSTANCE BY CONDITIONS OF MILD ALKALINITY\*

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During the course of a recent investigation, it was shown that auxin could be liberated by enzymes from the isolated leaf proteins of spinach (10). In order to utilize a more rapid method of hydrolysis, cytoplasmic proteins of spinach were refluxed with alkali. Approximately 10 times as much auxin was obtained by hydrolysis of the proteins with weak concentrations of sodium hydroxide as by the use of tryptic extract, a purified, non-crystalline pancreatic preparation of trypsin. Table I illustrates this difference.

Diffusion experiments (10) were undertaken to compare the relative molecular size of the auxin released by enzymes with the auxin released by alkali. Fig. 1 shows that the slopes of the curves for indoleacetic acid and the auxin released by alkali are similar, while both are steeper than the diffusion curve for auxin released by enzymes. Hence, the auxin obtained by alkali was suspected of being an artifact produced by chemical means. Since analyses of the cytoplasmic proteins from spinach made by Chibnall (4) showed a tryptophane content of 1.7 per cent, and since Thimann (7) has postulated the oxidative deamination of tryptophane by microorganisms to  $\beta$ -indoleacetic acid, it was considered that tryptophane in the leaf proteins might be converted to indoleacetic acid by the treatment with alkali, particularly in light of the similarity in diffusion rates mentioned above.

The *Avena* test offers a very sensitive method for the detection of exceedingly minute amounts of growth-accelerating substances. For instance, in the tests carried out in this laboratory, 0.9 c.mm. agar blocks containing 20  $\gamma$  of indoleacetic acid per liter will generally produce an average curvature of  $10^\circ$  when applied unilaterally to *Avena* coleoptiles (mean of twelve test plants). A simple calculation will show that approximately  $1.8 \times 10^{-5} \gamma$  of indoleacetic acid applied to one oat coleoptile is sufficient to cause a bending of  $10^\circ$ . Thus, it is possible to detect the presence of growth substances in amounts which would elude analysis by usual chemical methods. Accordingly, a study was instituted in which tryptophane was subjected to various conditions and analyses were made for growth-promoting substances formed under those conditions. Simi-

\* Paper from the Department of Botany of the University of Michigan, No. 808.

larly, proteins of animal origin containing or lacking tryptophane were studied for growth activity.

TABLE I

Comparison of Amount of Auxin Released from Cytoplasmic Proteins of Spinach by Enzymes and Alkali

The proteins were digested with enzymes for 72 hours at 37° in  $\text{KH}_2\text{PO}_4\text{-NaOH}$  buffer at pH 8.0.

Amount of protein	Type of digestion	Agar dilution	Average <i>Avena</i> curvature
mg.		ml.	degrees
40	Crystalline chymotrypsin	0.70	$1.5 \pm 0.5$
40	“ trypsin	0.70	$2.4 \pm 0.6$
40	Tryptic extract	0.70	$7.5 \pm 0.6$
		1.40	$4.0 \pm 0.5$
10	Boiling 12 hrs. with 5.0 ml. of 0.05 N NaOH	0.70	$20.6 \pm 0.4$
		1.40	$16.4 \pm 1.1$
		2.80	$11.5 \pm 0.8$

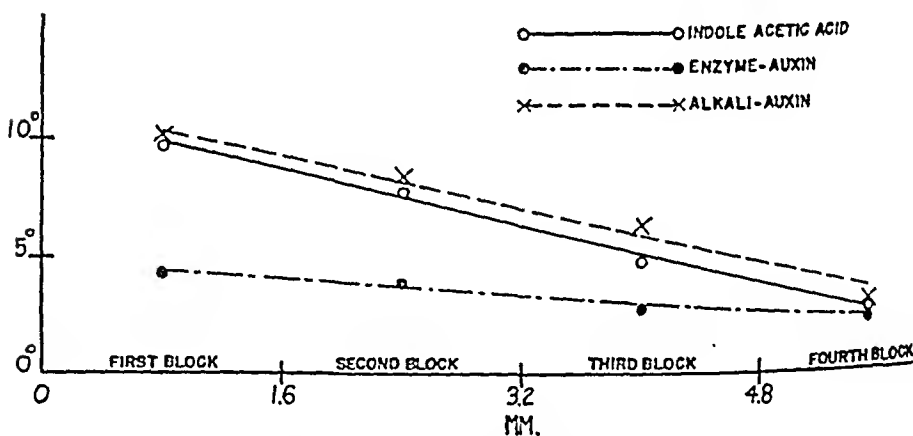


FIG. 1. The distribution of auxin in four agar blocks, each 1.6 mm. thick, after 90 minutes diffusion at 22°. The initial concentration of indoleacetic acid was 100  $\gamma$  per liter. The average *Avena* curvature is shown on the ordinate scale.

#### EXPERIMENTAL

The method used for the determination of auxin by the *Avena* technique has been described (9) and need not be detailed here. Six concentrations of indoleacetic acid, 10, 15, 20, 30, 40, and 50  $\gamma$  per liter of water, were made and assayed for each *Avena* experiment in order to obtain a concentration

curve for the conversion of experimental curvatures to units of indoleacetic acid. The standard deviation and standard error of the mean were calculated from the curvatures of eleven or twelve *Avena* plants, which was the minimum number of plants used for each determination. Individual curvatures which varied from the mean by more than 2.5 times the standard deviation were discarded when the average curvature was above  $5^\circ$ , and the means were recalculated; with smaller curvatures, 2.0 times the standard deviation was taken as a criterion for elimination. Curvatures listed in Tables I to V are in the proportionality range of dilution unless otherwise noted. All solutions after experimental treatment were adjusted to pH 4.5 (glass electrode), transferred to a separatory funnel, and the auxin was removed by shaking with two 75 ml. portions of ether recently distilled over  $\text{FeSO}_4\text{-Ca(OH)}_2$ . The ether was separated from the water and evaporated until only a few ml. remained. This was transferred to a small vial to which 1.5 per cent agar was then added and the material assayed for growth activity. An Eastman preparation of *l*-tryptophane was used in this study.

*Conversion of Tryptophane to Auxin under Varying Degrees of Alkalinity*—From a freshly made solution containing 50 mg. of tryptophane per 100 ml. of water, 2.5 ml. were pipetted into a digestion flask and 2.5 ml. of NaOH added, the latter being of such strength that the total volume of liquid resulting would have the normality represented in Table II. A condenser was placed in the neck of the flask and the assembly put into a boiling water bath for 7 hours (temperature of tryptophane-NaOH solution  $97.5^\circ$ ). At the completion of the alkali treatment, the pH was adjusted and the auxin removed with ether. As controls, 2.5 ml. of the above solution of tryptophane were diluted with 15 ml. of redistilled water to compensate for the water added in transferring from flask to beaker, and treated in an identical manner except that no heat or alkali was used. Table II shows the amount of growth substance produced by various concentrations of alkali. No auxin was obtained with 0.5 N NaOH; with decreasing concentrations of alkali, greater amounts of auxin were produced up to a maximum with 0.0005 N NaOH; thereafter, the auxin yield decreased, but auxin was produced even when the tryptophane was heated with redistilled water. Since controls gave no curvature, it is evident that the formation of growth substance from tryptophane can be attributed to the effect of alkali and heat. Another experiment under the same conditions showed the same trend.

*Effect of Temperature on Conversion of Tryptophane to Auxin by Strong Alkali*—On the assumption that relatively strong concentrations of alkali caused a conversion of tryptophane to some product other than auxin, a range of temperatures for short time intervals was tried. Over a water

TABLE II

*Conversion of Tryptophane to Auxin by Different Concentrations of Alkali*

All solutions of tryptophane except the controls were heated for 7 hours in a boiling water bath (see the text).

Concentration of NaOH	Agar dilution	Average <i>Arens</i> curvature	Indoleacetic acid equivalents
<i>N</i>	ml.	degrees	$\gamma \times 10^{-3}$
0.0000 (Control; not heated)	0.80	0	0
0.0000 " " "	0.80	0	0
0.0000 (Heated)	0.80	5.1 $\pm$ 0.7	12.4
	1.60	1.0 $\pm$ 0.3	14.7
0.00025	1.60	7.8 $\pm$ 0.6	31.2
	3.20	2.3 $\pm$ 0.9	36.0
0.0005	3.00	7.7 $\pm$ 1.2	49.5
0.0025	3.00	2.9 $\pm$ 0.6	32.4
0.005	2.00	3.9 $\pm$ 0.7	23.6
0.025	0.80	10.7 $\pm$ 1.4	20.3
	1.60	3.1 $\pm$ 0.6	17.6
0.05	0.80	13.7 $\pm$ 0.9	26.3
	1.60	5.3 $\pm$ 0.9	20.8
0.25	0.35	7.1 $\pm$ 0.6	5.3
0.5	0.35	0	0

TABLE III

*Amount of Tryptophane Converted to Auxin at Different Temperatures in Presence of Alkali*

Tryptophane (1.25 mg.) in contact with 0.25 *N* NaOH for 15 minutes.

Temperature	Agar dilution	Average <i>Arens</i> curvature	Indoleacetic acid equivalents	Average
$^{\circ}\text{C.}$	ml.	degrees	$\gamma \times 10^{-3}$	
22 (Control; no alkali)	0.80	0	0	0
22	0.80	18.6 $\pm$ 1.6	35.2	35.0
	1.60	10.0 $\pm$ 0.6	37.4	
	3.20	4.5 $\pm$ 0.5	32.6	
40	0.80	16.5 $\pm$ 0.9	31.2	38.9
	1.60	12.2 $\pm$ 0.5	45.6	
	3.20	5.5 $\pm$ 0.4	40.0	
60	0.80	13.8 $\pm$ 0.9	26.2	30.1
	1.60	9.4 $\pm$ 0.9	35.2	
	3.20	3.9 $\pm$ 0.6	28.8	
80	1.60	26.1 $\pm$ 1.8*	97.1	97.1
97.5	0.80	18.6 $\pm$ 1.0	35.2	38.0
	1.60	10.8 $\pm$ 0.6	40.8	
101.0	0.80	0	0	0

\* This curvature may be slightly outside of the dilution range. Since further dilutions could only raise the weight of auxin, the value of  $97 \times 10^{-3} \gamma$  can be taken as a conservative figure to indicate the trend of temperature effect.

bath, 1.25 mg. of tryptophane were allowed to stand in contact with 0.25 N NaOH for 15 minutes at different temperatures. The pH of the solution was then adjusted as before, and the auxin produced was immediately removed with ether. Table III shows the amount of auxin obtained at each temperature. It will be noticed that even with a short time exposure to 0.25 N NaOH at room temperature a relatively large amount of growth substance is produced, but a control not in contact with alkali gave no

TABLE IV

*Comparison of Amount of Auxin Released from Casein and Gelatin by Hydrolysis with Enzymes and Alkali*

The treatments with alkali were carried out for 12 hours at 100° or at 97.5°, while those with enzymes were for 72 hours at 37°.

Protein	Type of digestion	Agar dilution	Curvature	Indoleacetic acid equivalents
		ml.	degrees	$\gamma \times 10^{-3}$
Casein	0.05 N NaOH	1.50	10.6 $\pm$ 0.6	27.7
		3.00	4.5 $\pm$ 0.3	36.0
	0.05 " "	1.60	7.2 $\pm$ 1.0	28.5
		3.20	2.9 $\pm$ 0.7	28.8
	0.5 " "	3.20	14.9 $\pm$ 0.9	141
		6.40	8.8 $\pm$ 0.7	143
Gelatin	0.05 " "	0.35	12.2 $\pm$ 0.8	7.0
	0.05 " "	0.35	7.8 $\pm$ 0.9	4.3
	0.5 " "	0.35	8.3 $\pm$ 0.9	7.2
	0.5 " "	0.80	5.6 $\pm$ 0.9	11.7
Casein	Control	0.35	18.4 $\pm$ 1.0	15.2
	Trypsin	0.80	8.7 $\pm$ 1.2	16.0
	Chymotrypsin	0.80	13.3 $\pm$ 1.3	25.2
	Tryptic extract	0.80	11.0 $\pm$ 0.8	20.4
Gelatin	Control	0.35	4.7 $\pm$ 0.5	3.7
	Trypsin	0.80	0	0
	Chymotrypsin	0.80	0	0
	Tryptic extract	0.80	3.0 $\pm$ 0.7	5.2

curvature. No appreciable difference in auxin yield was obtained at 22°, 40°, 60°, and 97.5°. However, there was a marked increase in the amount of auxin at 80°. No curvature was obtained from material heated over an open flame at 101°. The same increase was noted at 80° upon repetition.

*Protein Digestion and Production of Auxin. By Alkali*—Since it was postulated that the tryptophane of proteins was a potential source of auxin because of its conversion by alkali to a substance similar to indoleacetic acid, two additional proteins were tested for growth hormone after being digested with NaOH. Casein and gelatin were selected, as the former

contains 1.5 per cent tryptophane and the latter contains none (2). Both proteins were extracted with redistilled ether which had been dried over sodium. The extract from 3 gm. of Eastman "desalted" gelatin obtained by prolonged extraction in a Soxhlet apparatus gave a very slight amount of curvature; extraction was continued until no more growth substance

TABLE V

*Conversion of Tryptophane to Auxin by Various Treatments*

Curvatures without standard error of the mean indicate concentrations of auxin too high for quantitative estimation.

Treatment	Agar dilution	Average Avena curvature
	ml.	degrees
2.50 ml. tryptophane solution (1.25 mg.) diluted with about 25 ml. water; pH adjusted to 4.6 and solution extracted with ether	0.80	0
	1.60	0
0.5 ml. tryptophane solution (0.63 mg.) dissolved in 0.5 ml. melted 3% agar	1.00	2.1 $\pm$ 0.6
2.5 mg. tryptophane dissolved in 0.80 ml. melted 1.5% agar; heated to 90° and stirred to insure uniform solution	0.80	18.7
	1.60	19.0
	3.20	22.5 $\pm$ 1.2
	6.40	17.0 $\pm$ 1.6
2.5 mg. tryptophane dissolved in 0.35 ml. cold water and 0.35 ml. melted 3% agar added; heated to 65° for 1 min. and stirred	0.80	20.2
	1.60	21.6
	3.20	20.5
	6.40	13.7 $\pm$ 0.8
1.25 mg. tryptophane dissolved in cold $\text{KH}_2\text{PO}_4$ buffer at pH 4.6 and about 25 ml. buffer added; solution immediately extracted with ether	0.80	15.8
	1.60	18.3
	3.20	19.3
	6.40	19.8
2.5 mg. tryptophane dissolved in 10 ml. water; pH adjusted to 10.5 and solution allowed to stand 48 hrs. at 37° with toluene as an antiseptic; pH adjusted to 4.5 and solution extracted with ether	0.35	20.3 $\pm$ 1.2
	0.75	13.9 $\pm$ 0.9
	1.50	9.8 $\pm$ 1.1

could be detected. 13 gm. of "vitamin-free" casein, on the other hand, were entirely free from ether-extractable growth hormone. 10 mg. of protein were weighed directly into a digestion flask and 5.0 ml. of either 0.5 N or 0.05 N NaOH were added. The protein was digested for 12 hours at 100° or in a water bath at 97.5°. At the end of the treatment, the pH of the solution was adjusted and the hormone removed with ether. In Table IV are given the results of the alkaline digestions. With gelatin, no

significant increase is apparent when digestion with 0.05 N NaOH is compared to digestion with 0.5 N NaOH. The yield in each instance was relatively small. Casein, however, not only gave much larger amounts of auxin, but there was a marked increase in the yield of auxin obtained with the more concentrated alkali.

*By Enzymes*—The method used for digestion by enzymes has been described (10). The same difference that was noted with alkali is evident between the two proteins when digested with enzymes. Gelatin yielded little, if any, auxin, depending upon the enzyme employed, while from casein was obtained a much larger quantity of growth substance. Moreover, the controls to which no enzyme was added show a similar variance between the two proteins. Whether or not the enzymes functioned in the liberation of auxin from these proteins is debatable considering the amount of hormone obtained from the controls. Of significance, however, is the large amount of auxin released from casein by strong alkali as compared to the amount released from casein by enzyme digestion; this pronounced difference is not apparent with gelatin.

*Tryptophane Instability under Other Conditions*—Table V illustrates the lability of tryptophane under relatively mild conditions of heat, pH, etc. The values for curvatures expressed in Table V are presented to describe certain characteristics of tryptophane rather than to provide finite values. Those curvatures not within the proportionality dilution range are listed to emphasize the relatively large amounts of auxin formed under these conditions. All of the treatments shown in Table V have been repeated and found to give essentially the same results. The table is self-explanatory and shows that the amino acid is unstable at 37° at pH 10.5, in the presence of hot agar, and in contact with cold  $\text{KH}_2\text{PO}_4$  buffer at pH 4.6, as evidenced by the large curvatures obtained.

#### DISCUSSION

Auxin is produced from tryptophane with 0.0005 N NaOH and not with 0.5 N NaOH after 7 hours contact with the alkali at near boiling temperatures. It seems reasonable to assume that tryptophane is degraded by alkali to indoleacetic acid, since a number of compounds containing the indole ring theoretically capable of arising from tryptophane, e.g. indolepropionic acid, tryptamine, indolecarboxylic acid, indole, skatole, etc. (3, 9), have been tested and found to be nearly devoid of growth activity as measured by the *Avena* test. Obviously, the activities demonstrated in this work are of the same order of magnitude as that of indoleacetic acid. Hence the formation of auxin from tryptophane by weak alkali can perhaps be explained on the basis of indoleacetic acid formation. However, the failure of strong alkali to form auxin is not easily explained. We have



considered the possibility that strong alkali converts tryptophane to a product incapable of giving a growth response. The experiments in which it was shown that auxin was formed from tryptophane which was in contact with strong alkali for only 15 minutes at low temperatures appear to make this hypothesis tenable. But a better proof of this explanation would lie in the degradation of the intermediate products of the reaction; i.e., conversion of inactive indolepropionic acid to active indoleacetic acid, and converting indoleacetic acid to inactive indolecarboxylic acid, indole, or skatole, etc., by strong base. This we have been unable to accomplish with either of the two compounds by boiling with 0.5 N NaOH for 6 hours. Indoleacetic acid is certainly stable under such conditions; although indolepropionic acid may be changed, it still fails to give a growth response. We have also considered that the amino group may contribute to the instability of the tryptophane molecule. With glycine as a potential source of amino nitrogen, equal molecular quantities of indoleacetic acid and glycine boiled with alkali failed to cause a loss of activity of the indoleacetic acid. Similarly, indolepropionic acid was not converted to a growth-active substance in the presence of glycine and alkali.

The problem would be simplified were it possible to adopt standard colorimetric procedures for the identification of tryptophane. Unfortunately, the reactions employed appear to be characteristic for the indole ring rather than specific for a particular compound (5), and even though a test for tryptophane after strong alkali treatment might indicate the presence of a compound containing the indole ring, one could still not be sure which one of the indole compounds was present. That an indole compound does remain after proteins are boiled with strong alkali is, after all, the basis of standard biochemical procedures for the determination of tryptophane.

Aside from the very interesting fact that animal proteins, after digestion, yield substances which stimulate growth when tested by oat plants, the much larger amount of hormone produced from casein by strong alkali compared to weak alkali and enzymatic hydrolysis is strikingly different from the amounts obtained in a similar manner from gelatin. The amino acids contained in casein which are theoretically absent from gelatin are valine, hydroxyglutamic acid, and tryptophane (2). In view of our previous findings on the tendency of tryptophane to be converted to auxin, it seems probable that the tryptophane contained in casein is the responsible factor. The larger quantity of auxin evolved from casein by heating with 0.5 N NaOH, when compared to yields by enzymes and 0.05 N NaOH and to the relatively constant amounts obtained from gelatin (irrespective of the mode of hydrolysis), may be interpreted as follows: the strong base hydrolyzes the protein molecule more than weak alkali;

the tryptophane so released is then acted upon by the alkali and decomposed to an auxin, presumably indoleacetic acid. Additional evidence in support of this explanation has been found by using zein, a protein which also lacks tryptophane. In preliminary experiments, almost identical amounts of auxin were obtained when this protein was boiled for 12 hours with 0.5 and 0.05  $\times$  NaOH. A study is now in progress at this laboratory which is designed to test the growth activity of those amino acids contained in gelatin, casein, and zein.

The data in Table V show that tryptophane is unstable under a variety of conditions, and still other conditions should be tried. Recently, a number of methods for the extraction of auxin from plant tissues have been suggested which incorporate the use of alkali (1, 6). It will be necessary to reconsider such results from the standpoint of tryptophane lability and rapid conversion to a plant growth substance. With one method of extraction (6), only 0.6 mg. of free tryptophane would have to be present in a kilo of wheat seeds to account for the increase in auxin reported. This has been calculated on the basis that 2.5 mg. of tryptophane under identical conditions of alkalinity yield 30.3  $\gamma$  of growth substance (Table V). Similarly, in another method boiling or autoclaving corn endosperm tissue with buffer solution at pH 9.0 to 10.0 is recommended. It is not improbable that tryptophane does occur as such in plants (8), and until it can be definitely shown that there is no conversion of this substance to auxin under the influence of alkali treatment, reported increases in auxin liberation must be viewed with misgiving. Cognizance should also be taken of the instability of tryptophane if nutrient solutions are to be made with (or with a source of) the amino acid and autoclaved with or without agar. The possibility exists that auxin produced from the amino acid may influence the specific reaction or phenomenon under investigation.

We realize that the information in this paper is not complete. Unfortunately, it has been necessary to terminate the problem at this stage. We are therefore presenting the data with the hope that they may be applied to the methods of plant hormone extraction and to the biological use of tryptophane.

We wish to express our appreciation to Dr. F. G. Gustafson and Professor H. H. Bartlett, Department of Botany, University of Michigan, for their criticism and interest in this work. We are indebted to Dr. H. B. Lewis, Head of the Department of Physiological Chemistry, University of Michigan, for his advice and for supplying the proteins used in this study. This work was performed while the authors held F. C. and Susan Eastman Newcombe Fellowships in plant physiology.

## SUMMARY

1. Tryptophane heated for 7 hours with NaOH varying in concentration from 0.25 to 0.00025  $N$  yields readily detected quantities of a plant growth hormone (auxin). Heating for 7 hours in distilled water also converts tryptophane to auxin.

2. When tryptophane is allowed to stand in contact with 0.25  $N$  NaOH for 15 minutes at room temperature, a large amount of auxin is formed; with increasing temperatures, still more auxin is formed, with maximal conversion at about 80°. At higher temperatures, auxin formation rapidly decreases.

3. Gelatin, a protein lacking tryptophane, produced about equal amounts of auxin whether hydrolyzed with 0.5 or 0.05  $N$  NaOH. Casein, a protein containing tryptophane, produced much larger amounts of auxin when hydrolyzed with 0.5  $N$  NaOH than with 0.05  $N$  NaOH. It is suggested that the tryptophane contained in proteins is a potential source of auxin when such proteins are digested with alkali.

4. Tryptophane is also rapidly converted to auxin under comparatively mild conditions of heat, pH, etc. Since recent methods for the extraction of auxin from plant tissues incorporate the use of alkaline conditions, it is suggested that these methods be reviewed in the light of the present observations on auxin formation from tryptophane under similar conditions.

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# THE DETERMINATION OF ASCORBIC ACID IN WHOLE BLOOD AND URINE THROUGH THE 2,4-DINITRO-PHENYLHYDRAZINE DERIVATIVE OF DEHYDROASCORBIC ACID\*

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In a previous paper (1) we have reported a new color reaction useful in the determination of vitamin C. When the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid is treated with 85 per cent sulfuric acid, a reddish colored product is formed which absorbs maximally at 500 to 550 and 350 to 380  $m\mu$ . The proportionality of the color obtained in this reaction is in excellent agreement with Beer's law in the ranges used (Fig. 1). In the procedure reported the dehydroascorbic acid osazone crystals are separated by centrifugation and washed before treatment for color production. These are time-consuming steps and they do not permit the development of a microprocedure. We have, therefore, used this new reaction to develop a direct method for the determination of vitamin C without removal of the reagent, 2,4-dinitrophenylhydrazine. In this paper we are reporting the application of this direct procedure to the determination of vitamin C in blood and urine.

In subvitaminosis C the ascorbic acid disappears more rapidly from the plasma than from the red cells, white cells, or platelets (2). Hence the vitamin C content of blood is of greatest interest when the determination is made upon whole blood. Almost all blood vitamin C determinations reported thus far have been upon plasma, for the reason that the oxidation-reduction methods in use are not applicable to whole blood analysis unless the oxyhemoglobin, which is an oxidant of ascorbic acid in deproteinizing procedures, is rendered inert by saturation with CO, as in the method of Butler and Cushman (3), or by reduction, as in the procedure developed by Kuether and Roe (4). The latter procedures are somewhat difficult for experimental work and are not adaptable to clinical use. Hence there is an outstanding need for a simple method for determining ascorbic acid in whole blood.

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We are indebted to Merck and Company, Inc., for contributions of ascorbic acid.

In our procedure a trichloroacetic acid filtrate of blood or urine is shaken with norit and filtered. The norit clarifies the solution and oxidizes the ascorbic acid to dehydroascorbic acid. Ascorbic acid is not oxidized quantitatively by norit unless the solution contains a reagent like acetic or trichloroacetic acid. Apparently the acetic acid is preferentially adsorbed on the norit and active oxygen is eluted in quantities sufficient for rapid oxidation. Norit filtrate is treated with 2,4-dinitrophenylhydrazine and thiourea for 3 hours at 37°. Thiourea is essential to produce a mildly reducing medium, as oxidants have been found to produce a slight color-

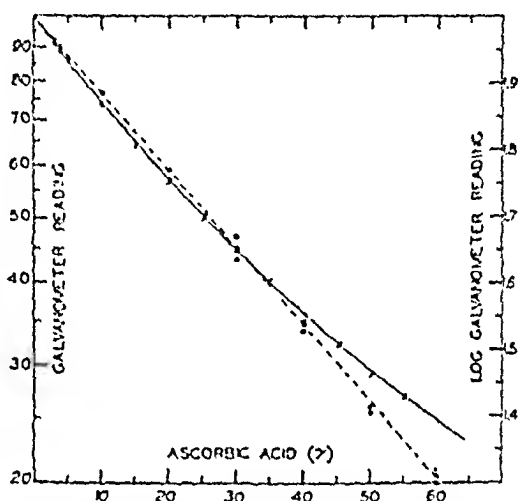


FIG. 1. Calibration curve made with the Evelyn photoelectric colorimeter with Filter 540. Solid line, curve obtained according to the procedure described in this paper (3 hours incubation at 37°); broken line, curve obtained when crystals are isolated after 21 hours at room temperature and then dissolved in 85 per cent  $H_2SO_4$ .

tion of the 2,4-dinitrophenylhydrazine solution at 37°. Color is produced by adding 85 per cent sulfuric acid. This method appears to be completely specific. It has exceptionally good precision and will permit determination of amounts down to 0.2  $\gamma$  in 4 cc. of filtrate.

#### Reagents—

**2,4-Dinitrophenylhydrazine reagent.** Dissolve 2 gm. of 2,4-dinitrophenylhydrazine in 100 cc. of approximately 9  $N$   $H_2SO_4$  (3 parts of  $H_2O$  to 1 part of concentrated  $H_2SO_4$ ) and filter.

**Acid-washed norit.** Place 200 gm. of norit in a large flask and add 1 liter of 10 per cent  $HCl$ . Heat to boiling. Filter with suction. Remove the cake of norit to a large beaker, add 1 liter of distilled water, stir up thoroughly, and filter. Repeat this procedure, until the washings give a

negative or very faint test for ferric ions. Dry overnight in an oven at 110–120°. Do not heat to high temperatures. We recommend washing the norit for complete protection against interference. Some grades of activated carbon may not require washing. To determine whether washing is necessary, a blank test may be run upon trichloroacetic acid washings of the carbon to be used; if the latter give no more color by this procedure than is obtained with trichloroacetic acid not passed through the carbon, then washing the carbon need not be carried out.

**Trichloroacetic acid.** Prepare a 6 per cent solution for blood work and a 4 per cent solution for urine analysis.

**85 per cent sulfuric acid.** To 100 cc. of distilled water add 900 cc. of concentrated  $\text{H}_2\text{SO}_4$ , sp. gr. 1.84.

**Thiourea solution.** Dissolve 10 gm. of thiourea in 100 cc. of 50 per cent (by volume) aqueous ethyl alcohol. We have found that this reagent keeps satisfactorily at least 2 months. It should be checked occasionally. It must readily reduce  $\text{HgCl}_2$  or  $\text{KMnO}_4$ .

#### *General Procedure*

**Preparation of Blood Filtrate**—In a 50 cc. centrifuge tube place 15 cc. of 6 per cent trichloroacetic acid. Add 5 cc. of whole blood (or plasma), drop by drop, and with a glass rod stir until a fine suspension is produced. Let stand at least 5 minutes, then centrifuge. To the supernatant fluid, add  $\frac{1}{2}$  teaspoonful (0.75 gm.) of acid-washed norit and vigorously shake or stir the latter through the liquid. Filter through a folded filter paper of about 9 cm. size.

**Preparation of Urine Filtrate**—To 1 volume of urine add 19 volumes of 4 per cent trichloroacetic acid. This dilution will serve for a range of 1 to 300 mg. of ascorbic acid per liter of urine. With urines high in vitamin C greater dilutions may be made, but dilutions of less than 1:20 should not be used. For each 40 cc. of diluted urine add 1 teaspoonful (1.5 gm.) of acid-washed norit. Shake vigorously and filter.

**Procedure for Norit Filtrate of Blood or Urine**—Place 4 cc. of norit filtrate of blood or urine in each of two matched photoelectric colorimeter tubes. Add to each tube 1 drop of the 10 per cent thiourea solution. Hold one tube in reserve for a blank and add to the other tube 1 cc. of the 2,4-dinitrophenylhydrazine reagent. Place the latter tube in a water bath at 37°. The bath must be well equipped by thermostat to maintain a constant temperature. Keep the tube immersed in the bath for exactly 3 hours. Remove and place in a beaker of ice water containing generous quantities of ice. Place the blank tube in the ice water also. To each of these tubes, while in the ice water bath, add very slowly 5 cc. of 85 per cent  $\text{H}_2\text{SO}_4$ . The sulfuric acid is added from a burette, a drop at a time, a step

TABLE I

*Calculation Chart for Use with Evelyn Photoelectric Colorimeter by Technique Outlined. Mg. of Ascorbic Acid against Galvanometer Reading*

G	0.00	0.25	0.50	0.75	G	0.00	0.25	0.50	0.75
100	0.0000	0.0000	0.0000	0.0000	59	0.0184	0.0182	0.0180	0.0179
99	0.0003	0.00022	0.00015	0.00008	58	0.0191	0.0189	0.0188	0.0186
98	0.0006	0.00052	0.00045	0.00038	57	0.0198	0.0196	0.0194	0.0193
97	0.0009	0.00082	0.00075	0.00068	56	0.0205	0.0203	0.0202	0.0200
96	0.0012	0.00112	0.00105	0.00098	55	0.0212	0.0210	0.0208	0.0207
95	0.0015	0.00142	0.00135	0.00128	54	0.0219	0.0217	0.0216	0.0214
94	0.0019	0.0018	0.0017	0.0016	53	0.0226	0.0224	0.0222	0.0221
93	0.0023	0.0022	0.0021	0.0020	52	0.0233	0.0231	0.0230	0.0228
92	0.0027	0.0026	0.0025	0.0024	51	0.0241	0.0239	0.0237	0.0235
91	0.0031	0.0030	0.0029	0.0028	50	0.0249	0.0247	0.0245	0.0243
90	0.0035	0.0034	0.0033	0.0032	49	0.0257	0.0255	0.0253	0.0250
89	0.0039	0.0038	0.0037	0.0036	48	0.0265	0.0263	0.0261	0.0259
88	0.0043	0.0042	0.0041	0.0040	47	0.0274	0.0272	0.0270	0.0267
87	0.0047	0.0046	0.0045	0.0044	46	0.0281	0.0282	0.0279	0.0276
86	0.0051	0.0050	0.0049	0.0048	45	0.0291	0.0292	0.0289	0.0287
85	0.0055	0.0054	0.0053	0.0052	44	0.0301	0.0301	0.0299	0.0296
84	0.0059	0.0058	0.0057	0.0056	43	0.0311	0.0312	0.0309	0.0307
83	0.0063	0.0062	0.0061	0.0060	42	0.0325	0.0322	0.0320	0.0317
82	0.0067	0.0066	0.0065	0.0064	41	0.0336	0.0333	0.0330	0.0328
81	0.0071	0.0070	0.0069	0.0068	40	0.0347	0.0344	0.0342	0.0339
80	0.0075	0.0074	0.0073	0.0072	39	0.0359	0.0356	0.0353	0.0350
79	0.0079	0.0078	0.0077	0.0076	38	0.0371	0.0368	0.0365	0.0362
78	0.0083	0.0082	0.0081	0.0080	37	0.0383	0.0380	0.0377	0.0374
77	0.0087	0.0086	0.0085	0.0084	36	0.0396	0.0393	0.0390	0.0386
76	0.0091	0.0090	0.0089	0.0088	35	0.0410	0.0407	0.0403	0.0400
75	0.0095	0.0094	0.0093	0.0092	34	0.0424	0.0420	0.0417	0.0413
74	0.0100	0.0099	0.0097	0.0096	33	0.0439	0.0435	0.0431	0.0428
73	0.0105	0.0104	0.0103	0.0101	32	0.0455	0.0451	0.0447	0.0443
72	0.0110	0.0108	0.0107	0.0106	31	0.0472	0.0468	0.0463	0.0459
71	0.0115	0.0114	0.0113	0.0112	30	0.0489	0.0485	0.0481	0.0476
70	0.0120	0.0119	0.0117	0.0116	29	0.0507	0.0502	0.0498	0.0493
69	0.0125	0.0124	0.0123	0.0121	28	0.0526	0.0521	0.0517	0.0512
68	0.0130	0.0128	0.0127	0.0126	27	0.0547	0.0542	0.0536	0.0531
67	0.0135	0.0134	0.0133	0.0132	26	0.0569	0.0564	0.0558	0.0553
66	0.0141	0.0139	0.0138	0.0136	25	0.0593	0.0587	0.0581	0.0575
65	0.0147	0.0145	0.0144	0.0143					
64	0.0153	0.0151	0.0150	0.0148					
63	0.0159	0.0157	0.0155	0.0154					
62	0.0165	0.0163	0.0162	0.0160					
61	0.0171	0.0169	0.0168	0.0166					
60	0.0177	0.0175	0.0174	0.0172					

which requires about 1 minute. (Do not use stop-cock grease. Use  $H_2SO_4$  for lubricating the stop-cock.) It is very important not to allow the temperature of the solution to be raised appreciably, as this will bring

about a charring of sugars, or other organic matter, and thus a positive error will be introduced. Finally to the blank tube add 1 cc. of the 2,4-dinitrophenylhydrazine reagent. Both tubes are shaken thoroughly under the ice water to obtain complete mixing and are then removed to a rack. After 30 minutes wipe the tubes dry and clean and read in a photoelectric colorimeter. The correct filter is one transmitting maximally at 540  $m\mu$ . To make the reading, place the blank tube in the colorimeter and set the galvanometer at 100 if the Evelyn type of colorimeter is used, or at zero if a null point instrument (Klett-Summerson) is employed; then take the reading of the unknown. If an Evelyn colorimeter with a 540  $m\mu$  filter has been used, Table I may be employed for calculation. As 4 cc. of filtrate were taken, the calculation will be as follows:

*For Blood, 1:4 Dilution*—(Mg. from Table I)  $\times 100$  = mg. per 100 cc. of blood.

*For Urine, 1:20 Dilution*—(Mg. from Table I)  $\times 5000$  = mg. per liter of urine.

If a null point colorimeter is used, appropriate standards must be run along with the unknown tubes, or a calibration curve and chart must be constructed. To do this make up standard solutions of ascorbic acid varying from 0.25 to 15  $\gamma$  per cc. in 4 per cent trichloroacetic acid. Treat with norit, filter, and make the determinations exactly in the same way as described above.

The above outlines this procedure in its simplest form. One may run duplicates as desired. Actually the precision is so good that a single determination may be relied upon except for precise or critical investigations. If an Evelyn colorimeter is used, a constant center setting for the blank tube is established with a little experience, and after that the blank need not be run, except occasionally, or when new reagents are prepared. Twelve to thirty tubes are conveniently run simultaneously; hence one may analyze a large number of bloods or urines at the same time.

This determination necessitates a photoelectric colorimeter in order that a compensatory adjustment for the yellow color in the reagent blank may be made.

#### DISCUSSION

*Specificity*—We have established the specificity of this method by several types of experiments.

First, we analyzed the blood and urine of guinea pigs with acute scurvy. We obtained whole blood values ranging from 0.04 to 0.13 mg. per 100 cc. and urine values of 1.0 to 3.0 mg. per liter. One urine sample collected post mortem from the bladder of a guinea pig that died with scurvy gave a completely negative value for ascorbic acid. Inasmuch as one may not expect complete disappearance of vitamin C from the red cells, white cells, and platelets of the blood, or from the urine of guinea pigs at the onset of



acute symptoms of scurvy, these findings are interpreted as good evidence for the specificity of this method.

A second type of evidence is obtained by an analysis of interfering substances. In this method interference would be expected from aldehydes or ketones, which couple with 2,4-dinitrophenylhydrazine. Such compounds as pyruvic acid and acetoacetic acid readily couple with 2,4-dinitrophenylhydrazine but their derivatives do not react with sulfuric acid under the conditions of this reaction and sulfuric acid solutions of these derivatives do not absorb in the 540  $m\mu$  region in which the color obtained with the ascorbic acid derivative is read.

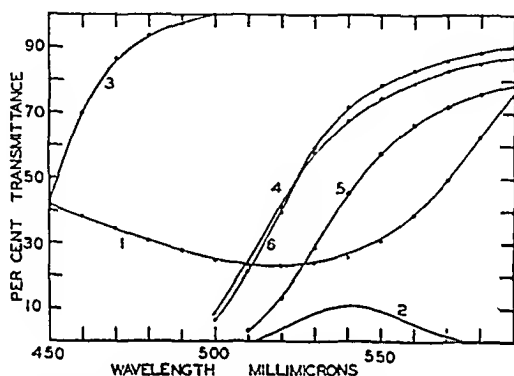


FIG. 2. Transmittance curves of derivatives of 2,4-dinitrophenylhydrazine obtained with the Coleman photoelectric spectrophotometer. Curve 1 represents the dehydroascorbic acid derivative; Curve 2, the transmission curve of Filter 540 (Evelyn); Curve 3, the blank with reagents; Curve 4, the glucose derivative; Curve 5, the fructose derivative; Curve 6, the xylose derivative.

The absorption spectra of sulfuric acid solutions of the derivatives formed with pentoses, hexoses, and glucuronic acid show that the latter might interfere slightly in this method (Fig. 2). However, the concentrations at which these substances yield interference are very considerably greater than those found in blood and urine filtrates. As shown in Table II, glucose begins to interfere when there are present 1.5 mg. per cc. This means that in blood filtrates of a 1:4 dilution interference will not occur until a blood containing 600 mg. per 100 cc. is encountered; and even in diabetic bloods with glucose concentrations ranging from 600 to 1200 mg. per 100 cc. the amounts of additive error would be only 0.008 to 0.03 mg. per 100 cc. In urine the substance most likely to interfere is glucuronic acid. This substance exists in the urine principally in the conjugated form but one must consider that hydrolysis may occur in a solution as strongly acid as that used in the coupling process of this method. As shown by Table II,

free glucuronic acid begins to interfere when there is present 0.125 mg. per cc., or 125 mg. per liter. Since the urine is diluted 20 times, or more, in this method, one may not expect interference until a urine containing 2500 mg. of free glucuronic acid per liter is encountered. The latter concentration of glucuronic acid in urine would be observed only rarely, if at all. In blood the total glucuronic acid concentration has been reported to be 10 to 25 mg. per cent (5). Table II shows that interference may not be expected in blood until a concentration of 50 mg. per cent is reached, and analysis of bloods with this content of glucuronic acid would show a plus error of only 0.04 mg. per 100 cc. It thus appears that interference from glucuronic acid in blood and urine analysis may be expected to be negligible.

TABLE II

*Data Showing Amount of Plus Error That May Occur in Analysis of Solutions Containing Glucose, Xylose, Fructose, or Glucuronic Acid*

The results are given in mg. per cc.

Quantity analyzed	Equivalent as ascorbic acid			
	Glucose	Xylose	Fructose	Glucuronic acid
0.125				0.00010
0.25				0.00022
0.50			0.00002	0.00039
1.00		0.00002	0.00006	0.00092
1.50	0.00002	0.00005	0.00012	
2.25	0.00005	0.00010	0.00014	
3.00	0.00008	0.00020	0.00017	

Of outstanding importance is the fact that one may easily determine whether or not interference from substances that couple with 2,4-dinitrophenylhydrazine exists in this method. This may be done by the simple expedient of determining the amount of ascorbic acid in norit filtrates of varying dilutions. As shown in Table II, when glucuronic acid, fructose, xylose, or glucose is present in amounts sufficient to couple, ascorbic acid yields more color than these substances by amounts averaging approximately as follows: glucuronic acid 1200 times, fructose 17,000 times, xylose 29,000 times, glucose 52,000 times. From these data it is obvious that one may dilute a filtrate until no reaction is obtained with these possible interfering substances and still get a satisfactory reaction with ascorbic acid. In a practical test one would expect that dilution of a filtrate would give values lower in proportion to the dilution made, if interference is present; and if one gets the same values upon dilution, one

would assume that interference is not present. When filtrates of the same blood are made by diluting 1:4, 1:8, and 1:16, or filtrates of the same urine are prepared by diluting 1:20, 1:40, and 1:80, and these varying dilutions are analyzed by our method, the same value is obtained in the three dilutions of the blood or the urine, within limits of satisfactory precision. Thus, this method not only appears to be completely specific by theoretical considerations but it is also capable of being simply and rapidly tested for interfering substances in any blood or urine.

As this is a very sensitive reaction, one must guard against substances that react with the reagents. We have found that oxidizing agents such as  $\text{Fe}^{+++}$  ions and  $\text{H}_2\text{O}_2$  will produce an interfering color with 2,4-dinitrophenylhydrazine. On the other hand, reducing agents such as thiourea,  $\text{Fe}^{++}$ , or  $\text{Sn}^{++}$  ions do not interfere. In order to protect against interference by oxidizing agents we introduce a small amount of thiourea into the reaction mixture. This produces a mildly reducing medium which does not slow down the coupling appreciably and is a complete protection against the slight interference from oxidants that might occur. The nitrite is washed with acid to remove traces of metals in the elementary form, also to diminish the concentration of Fe ions.

*Accuracy and Precision*—We have added ascorbic acid to blood and urine and obtained recoveries by this method ranging from 96 to 104 per cent. The precision of this method is excellent. In an experiment in which ten analyses of the same blood filtrate were carried out, the mean value was 0.71 mg. per 100 cc., the standard deviation was  $\pm 0.01$ , the probable error of a single determination was  $\pm 0.007$ , and the maximum deviation was 0.04 mg. per 100 cc.

*Mechanism of Reaction*—The red product formed in this procedure appears to be a new compound for the following reasons. Solutions of the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid in alcohol, glacial acetic acid, and dilute sulfuric acid are brown in color. When the concentration of sulfuric acid is raised to 60 to 85 per cent, a deep red color is formed. Addition of excess water causes the separation of a brownish compound which has the same appearance and absorption spectrum as the original derivative and yields a red solution when mixed again with 85 per cent sulfuric acid. The reaction is therefore reversible and it is suggested that the mechanism is a dehydration.

*Stability of Color*—This color is quite stable. Prepared from blood filtrate, it showed no change in 40 minutes and a maximum fading of 2.25 points in the galvanometer reading in 18 hours on standing in an open tube.

#### SUMMARY

1. A new method for the determination of ascorbic acid in whole blood or plasma and in urine has been developed. In this method dehydro-

ascorbic acid is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative is treated with  $H_2SO_4$  to produce a newly observed color which is measured in a photoelectric colorimeter.

2. This method is rapid and it possesses a high degree of specificity and precision.

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# STUDIES ON THE METABOLISM OF ZINC WITH THE AID OF ITS RADIOACTIVE ISOTOPE

## I. THE EXCRETION OF ADMINISTERED ZINC IN URINE AND FECES

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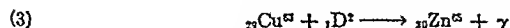
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Although investigations of the distribution of administered zinc in the body have been carried out (1-3), they have required the introduction into the animal of relatively large amounts of the salt of this metal. While these studies are significant in respect to the amounts administered, the extent to which they depict the metabolism of zinc in the normal physiological state is difficult to determine.

In the present investigation the excretion of zinc was studied in the dog and mouse by means of its radioactive *isotope*. The use of radiozinc permitted the intravenous administration of minute amounts of the salt of the metal. Since the total zinc content of the body was not increased appreciably by the introduction of the labeled zinc, the excretion of  $\text{Zn}^{65}$  may be regarded as representing the endogenous picture of zinc excretion.

### EXPERIMENTAL

Radiozinc ( $\text{Zn}^{65}$ ) was removed from the surface of the copper deflector plate of the 60 inch cyclotron. It was produced during the course of numerous bombardments with both protons and deuterons. The reactions involved are shown in the following equations. According to Livingood and Seaborg (4),  $\text{Zn}^{65}$  is produced by the reaction shown in Equation 1 plus either one of those shown in Equations 2 and 3.



The copper deflector plate was washed with cotton moistened with concentrated nitric acid. The cotton was then treated with HCl and the solution obtained evaporated to dryness. To insure complete removal of nitric acid, HCl was added to the dried material and the mixture again brought to dryness. 0.5 N HCl was added and the solution saturated with  $\text{H}_2\text{S}$ . The acid-insoluble sulfides were filtered out. When large concen-

trations of copper were present, it was found necessary to repeat the  $\text{H}_2\text{S}$  treatment several times to effect the complete removal of the copper. The filtrate was evaporated to dryness and thus freed of  $\text{H}_2\text{S}$ . The residue so obtained was dissolved in a small quantity of 0.5 N HCl and the solution transferred to a separatory funnel. 0.5 ml. of 20 per cent citric acid was added and the solution made slightly alkaline with concentrated  $\text{NH}_4\text{OH}$ . The zinc was then removed by extraction with a chloroform solution of dithizone. The chloroform phase was washed with dilute ammonia water to remove excess dithizone. The zinc was extracted from the chloroform layer with 0.1 N HCl.

Since the most likely contaminants of zinc obtained by this method of extraction are cobalt, nickel, lead, and cadmium, tests were made at this point to determine their presence in the 0.1 N HCl solution. A few mg. of  $\text{ZnCl}_2$  were added as carrier to each of a series of small aliquots of the 0.1 N HCl solution. A small quantity of either  $\text{CoCl}_2$  or  $\text{NiCl}_2$  or  $\text{Pb}(\text{NO}_3)_2$  or  $\text{CdCl}_2$  was then added to each aliquot and these metals separated from the zinc. The cadmium and lead were precipitated as sulfides and the cobalt and nickel as hydroxides. Each precipitate was redissolved and reprecipitated to reduce adsorption or occlusion of zinc; the precipitates were then tested for radioactivity. In no case was radioactivity found in the precipitates of cadmium, cobalt, nickel, or lead. Since radioactive cadmium, lead, cobalt, or nickel would have been precipitated with their respective non-radioactive isotopes, it was concluded that the solutions of radioactive zinc were free of the radioactive isotopes of cadmium, nickel, cobalt, and lead.

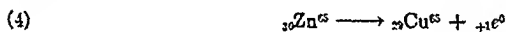
The 0.1 N HCl solution was then taken to dryness and the  $\text{ZnCl}_2$  dissolved in water. The zinc content of this solution was determined colorimetrically by shaking it with an ammoniacal chloroform solution of dithizone and comparing the color so produced with standard solutions prepared in the same manner. The ammoniacal chloroform solution of dithizone containing zinc as an organic complex was then subjected to (1) evaporation, to get rid of the chloroform and ammonia, and (2) ashing at  $450^\circ$ , to get rid of organic material. The Zn was finally obtained as a solution of its chloride.

*Injection and Care of Animals.* *Dogs*—Adult dogs weighing from 3.5 to 5.2 kilos were injected intravenously with the solution of radioactive  $\text{ZnCl}_2$ . The animals were placed in individual metabolism cages where the feces were caught on a wire screen and the urine collected in a glass bottle. Specimens of urine and feces were removed at intervals.

*Mice*—The large ventral tail veins of 3 month-old mice weighing 18 to 23 gm. were dilated by placing the tails in a beaker of warm water for 1 to 2 minutes. The  $\text{ZnCl}_2$  solution was diluted with a large volume of

normal saline; 0.10 cc. was the total volume injected into the dilated tail vein of each mouse. The mice were then placed in glass beakers which served as metabolism cages. Filter paper folded in the shape of a cone was placed on the bottom of the beaker and covered with  $\frac{1}{4}$  inch mesh screen. The urine was absorbed by the paper, where it soon dried. The feces rolled off toward the sides of the beaker. The feces were carefully removed from the beaker. The beaker was then washed and the washings transferred to a casserole containing the filter paper, where it was evaporated to dryness.

*Analysis of Excreta. Mice*—The fecal material was carefully removed from the filter paper containing the dried urine by means of fine forceps and a camel's hair brush. The feces and the filter paper containing the dried urine were ashed separately at  $450^{\circ}$  in an electric furnace. The ash was transferred to squares of blotter paper, which were then wrapped in cellophane, and its radioactivity determined by a Geiger-Müller counter.  $\text{Zn}^{65}$ , in its decay, emits both positrons and  $\gamma$ -rays, as shown by the following reactions (4).



Emission of positrons, as shown in Equation 4, is of secondary importance to the electron capture shown in Equation 5. The positrons emitted have relatively low radiation energies (5). In the case of such low energy values, self-absorption might be expected to complicate the measurement of the activity of samples yielding different ash weights. Under the conditions employed here, however, it was found that varying amounts of ash containing the same quantity of radioactive zinc yielded approximately the same number of counts per minute on the Geiger-Müller counter. This was taken to mean that the materials used in wrapping the samples together with the counter tube were of such thickness as to absorb effectively the soft particle radiation (positrons). Thus in the measurements of  $\text{Zn}^{65}$  only the hard penetrating  $\gamma$ -rays were counted and no correction was necessary for self-absorption of particles within the sample. Counts thus obtained were compared with those of blanks made from the stock solution of  $\text{Zn}^{65}$ . The radioactivity found in each sample of urine and feces is expressed as a percentage of the administered dose.

*Dogs*—Owing to the larger quantities involved, the analyses of dog excreta presented a somewhat different problem from those of the mice. The samples of urine and feces were dried and ashed in the electric muffle at  $450^{\circ}$  for 12 to 18 hours. If at this time appreciable amounts of unashed material remained, the soluble portion was dissolved in HCl and filtered off, the unashed fraction returned to the muffle, and the ashing continued.



Occasionally small quantities of the larger samples were not completely ignited even after the second ashing. In these cases the small amounts of HCl-insoluble material were filtered out and a separate determination of its activity made. The HCl solution was filtered into a volumetric flask and diluted to volume. Suitable aliquots were pipetted into beakers and diluted to 25 to 30 cc. Approximately 30 mg. of Zn (as the chloride) were added and the solution nearly neutralized with dilute NaOH. The zinc was precipitated as the carbonate by the addition of a saturated solution of  $K_2CO_3$ . Care was taken not to add a large excess of the carbonate solution. If the solution was too acidic, a considerable amount of foaming occurred upon the addition of the carbonate. The beakers were either placed on a steam bath for a few minutes or allowed to stand overnight, to aid coagulation of the precipitate. The precipitate was filtered through a 5.5 cm. filter paper. The paper was removed from the filter funnel and the edges trimmed to form a rectangle. The precipitate was spread over the surface of the filter paper and dried by means of radiant heat from an electric light bulb. The sample was then wrapped with 2 inch Scotch tape. Its radioactivity was determined as described above.

### Results

*Mice*—The results obtained in mice are recorded in Fig. 1. Samples of urine and feces at each time interval were obtained from a group of three to four mice kept in a single chamber. The values recorded in Fig. 1 are the averages for a single mouse. Observations were extended over a period of 170 hours. Each mouse received intravenously a total of 0.33  $\gamma$  of labeled zinc, an amount that is negligible with respect to the amount of zinc already contained in the animal.

At the end of 170 hours, over 50 per cent of the injected  $Zn^{65}$  was eliminated by way of the gastrointestinal tract. The most rapid excretion by this path occurred during the first 10 hours; at the end of this time approximately 20 per cent of the administered radiozinc was found in the feces.

Injected radiozinc made its appearance early in the urine of mice. 0.2 per cent of the injected dose was present in the urine as early as 2 hours. A total of 2 per cent of the administered  $Zn^{65}$  was found in the urine by 170 hours.

*Dogs*—The results obtained on two dogs are recorded in Fig. 2. Dog XV weighed 4.8 kilos and received intravenously 5.7  $\gamma$  of labeled zinc, whereas Dog XVI, which weighed 4.0 kilos, was injected with 6.5  $\gamma$  of labeled zinc. The excretion of  $Zn^{65}$  was measured during a period of 15 days.

It required 15 days for approximately 25 per cent of the injected  $Zn^{65}$  to be excreted in the feces of the dog. Although the slopes of the curves for feces in Fig. 2 are smaller at the later intervals than at the earlier ones,

it should not be inferred that the total amount of zinc eliminated by the gastrointestinal tract is less during the earlier than during the later intervals.

The specific activity of the circulating zinc is higher in the earlier than in the later intervals after the injection of  $Zn^{65}$ . This probably accounts for the change with time in the slopes of the fecal curves shown in Fig. 2.

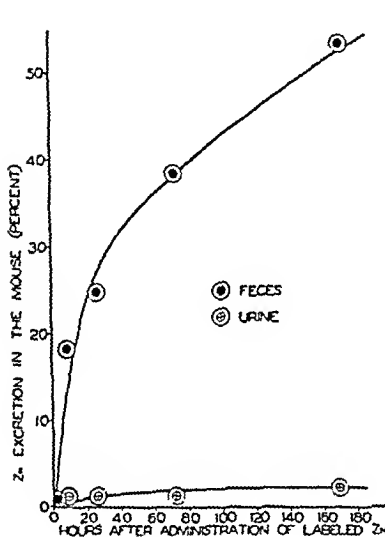


FIG. 1

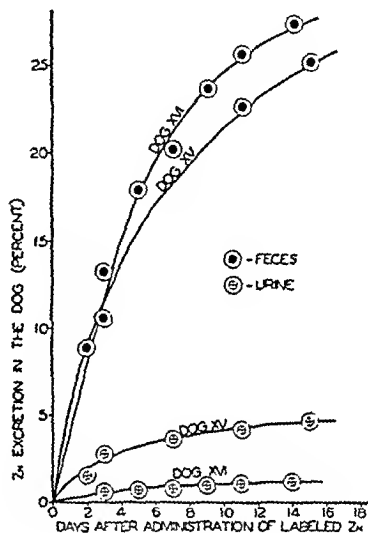


FIG. 2

FIG. 1. The excretion of  $Zn^{65}$  in the urine and feces of the mouse. Each point represents the average value for a single mouse. Each mouse received 0.33  $\gamma$  of labeled zinc intravenously. The mice weighed 18 to 23 gm.

FIG. 2. The excretion of  $Zn^{65}$  in the urine and feces of the dog. Dog XV weighed 4.8 kilos and received 5.7  $\gamma$  of labeled zinc intravenously. Dog XVI weighed 4.0 kilos and received 6.5  $\gamma$  of labeled zinc intravenously.

Less than 5 per cent of the  $Zn^{65}$  was excreted in the urine of Dog XV during the first 15 days after its administration. Even less was excreted in the urine of Dog XVI during this interval.

As already pointed out, the total amount of zinc injected is negligible as compared with the total zinc content of the dog; and hence it is reasonable to believe that the excretion of zinc observed here is an index of the endogenous excretion of zinc.

Our thanks are due to Professor E. O. Lawrence and members of the Radiation Laboratory for use of the deflector plate of the cyclotron from

which the radiozinc was obtained. The assistance of Dr. P. R. Stout in the separation of the  $\text{Zn}^{65}$  is gratefully acknowledged.

#### SUMMARY

The excretion of intravenously injected radiozinc into the urine and feces of the dog and mouse was investigated. The use of the radioactive isotope permitted the injection of minute amounts of zinc, amounts that were negligible when compared with the total amounts of zinc already contained in the animal.

1. A large fraction of the  $\text{Zn}^{65}$  appeared in the feces. In the mouse, as much as 50 per cent was eliminated by way of the gastrointestinal tract in 170 hours. In the dog, about 25 per cent was found in the feces at the end of 12 to 14 days.

2. Labeled zinc appeared early in the urine of both mice and dogs and continued to be excreted throughout the periods of observation; namely, 170 hours in the case of the mice, and 15 days in the case of the dogs. The amounts of  $\text{Zn}^{65}$  eliminated by this route were small, compared with those by way of the feces. A total of 2 per cent of the administered radiozinc was found in the urine of the mouse at the end of 170 hours. In 15 days the dog eliminated 1.2 to 4.7 per cent of the injected  $\text{Zn}^{65}$  in the urine.

3. The results obtained here are interpreted to mean that a large fraction of the body zinc is eliminated by way of the gastrointestinal tract.

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## PYRUVIC ACID

### II. THE DETERMINATION OF KETO ACIDS IN BLOOD AND URINE\*

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Of the many methods used for the determination of pyruvic acid, the most sensitive are those which are based upon the reaction of this acid with the nitrophenylhydrazines. These methods are not specific for pyruvic acid, but they are highly specific for keto acids as a group. The object of this paper is to point out how present procedures can be modified for identification and determination of the individual keto acids.

The principles underlying these methods were first described in 1913 by Dakin and Dudley (1). These authors noted that 4-nitrophenylhydrazinopropionic acid and the 4-nitrophenylhydrazones of pyruvic, glyoxylic, and phenylglyoxylic acids could be separated from other hydrazones either by treatment with a 10 per cent solution of sodium carbonate or by their differential solubility in alcohol. The salts of the hydrazones, if present in sufficient quantity, could be reprecipitated by acidification of the alkaline solution. Dakin and Dudley further noted that the "merest trace" of the hydrazones of glyoxal, methylglyoxal, and phenylglyoxal in sodium hydroxide solution, best with NaOH in alcohol, gave blue or purple colors which faded to red or brown; on the other hand, all of the acid hydrazones, including that of phenylglyoxylic acid, gave an intense red coloration with NaOH solution or with NaOH in alcohol.<sup>1</sup> These principles have since been applied by Neuberg (5-7), Barrenscheen and Dregus (8), Case (9), Peters and Thompson (10), Pi-Suñer and Farrán (11), Lu (12), Larsson and Liljedahl (13), and Bueding and Wortis (14).

Lu (12) has applied the procedure to the determination of pyruvic acid

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The experimental data were taken from a thesis submitted by Gladys E. Haugen to the Department of Physiological Chemistry of Northwestern University Medical School in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School of Northwestern University, 1942.

<sup>1</sup> That derivatives of 4-nitrophenylhydrazine yield violet or red solutions in sodium hydroxide was first noted by Bamberger (2). Hyde (3), who first prepared the pyruvic acid hydrazone and many other hydrazine derivatives, and who described their color reactions in alkali, failed to record the effect of alkali on the pyruvic acid derivative. Other 4-nitrophenylhydrazones were prepared by Dakin (4), all of which, including the glyoxylic acid derivative, gave deep red colors in alkali.

in small samples of blood. This has been modified somewhat by Bueding and Wortis (14) and others (15, 16). In these methods the trichloroacetic acid filtrate (8) is allowed to react 10 minutes with 1 mg. of 2,4-dinitrophenylhydrazine in 2 N HCl, after which the solution is extracted 3 times with small volumes of ethyl acetate. The combined extracts are then reextracted 3 times with small volumes of 10 per cent solution of  $\text{Na}_2\text{CO}_3$ . The combined alkaline extracts are extracted once more with ethyl acetate, after which they are made strongly alkaline with NaOH and the intensity of the resulting red color is determined in a photoelectric colorimeter. The reaction with hydrazine and the extractions are carried out in test-tubes. The phases are mixed by blowing air or nitrogen through a Wright pipette (a glass tube drawn to a capillary point), and one of the phases is removed after each extraction by means of the pipette which is fitted with a rubber bulb.

Although the Lu method represents a great advance in technique, it still retains one of the features of the older procedures which could be eliminated without loss of accuracy or specificity; namely, the *complete* extraction of the hydrazones by means of ethyl acetate and the subsequent *complete* extraction of the acid hydrazones with strong (10 per cent) carbonate solution. The seven extractions require much time, and the many transfers of solution from one tube to another (three test-tubes are needed) require the careful attention of the analyst. We have found that one extraction of the hydrazones from the reaction mixture and one reextraction with carbonate are sufficient. Only one test-tube is required; this eliminates the laborious transfer of extracts from one tube to another.

In addition to its simplicity, this procedure favors the differential extraction of hydrazones. For example, 8 cc. of ethyl acetate, when well mixed with the 4 cc. of reaction mixture, extract approximately 81 per cent of the pyruvic acid hydrazone, 79 per cent of the  $\alpha$ -ketoglutaric acid hydrazone, and 89 per cent of oxalacetic acid hydrazone. On the other hand, toluene extracts 93 per cent of pyruvic acid hydrazone, 29 per cent of  $\alpha$ -ketoglutaric acid hydrazone, and only 18 per cent of oxalacetic acid hydrazone. The specificity can therefore be greatly enhanced by the proper choice of solvent. These differences are not so apparent when the reaction mixture is extracted *completely*, as in older procedures.

As was shown by Bamberger, Hyde, and Dakin and Dakin and Dudley, all of the nitrophenylhydrazones possess the property of forming deeply colored water-soluble salts in sodium hydroxide. Thus, a solution of any of the hydrazones, methylglyoxal hydrazone for example, in benzene or other suitable solvent is readily extracted by means of strong alkali. When the extraction is carried out with a 10 per cent solution of carbonate, the hydrazones of glyoxals and aldehydes are extracted only slightly,

while the hydrazones of keto acids are extracted almost quantitatively. Since the hydrazones of glyoxals and aldehydes form some salt even in 10 per cent carbonate solution, and thus become more soluble in the aqueous phase, their effect can be minimized by reducing the number of extractions.

Besides the simplified Lu procedure for the determination of keto acids, we shall present also a method<sup>2</sup> for the determination of "total hydrazones" in blood which should prove useful not only as a rapid clinical method, but also as a means of determining (by difference) the concentration of reactive neutral keto compounds (aldehydes, ketones, trioses).

### *Method*

#### *Reagents—*

*Protein precipitants.* A 10 per cent solution of trichloroacetic acid is prepared at frequent intervals and kept in the refrigerator when not in use. Old solutions, or solutions which have stood overnight in a warm room, may contain considerable quantities of a substance, presumably a keto acid, which yields increased and variable blanks.

A 10 per cent solution of metaphosphoric acid is freshly prepared each week and kept in the refrigerator when not in use.

#### *Lloyd's reagent.*

*Solvents.* Xylene, toluene, and benzene are recommended for the determination of pyruvic acid. Ethyl acetate<sup>3</sup> or other non-specific solvents such as ethyl ether and caprylic alcohol are useful in the determination of total keto acids.

*Keto acid standards.* Either freshly distilled pyruvic acid or lithium pyruvate, prepared according to the directions of Wendel (18), may be used. Enough  $H_2SO_4$  is added to bring the final acidity to approximately 0.1 N. The  $\alpha$ -ketoglutaric acid standard is similarly prepared. None of these standards when kept in the refrigerator has shown evidence of deterioration during a period of at least 6 months (19).

5 cc. of freshly diluted standard solution, containing from 0.25 to 6.0 mg. of pyruvic acid per 100 cc., are added to 25 cc. of acid precipitant. The determinations are then carried out as described later under "Extraction method."

<sup>2</sup> A similar method has been described by Sealock (17).

<sup>3</sup> Ethyl acetate should be anhydrous and of c.p. or reagent quality. The used reagent is extracted three times with 0.1 volume each of a saturated solution of calcium chloride (approximately 75 per cent at 30°). Anhydrous sodium sulfate is then added (200 gm. to each liter of ethyl acetate), and the mixture is allowed to stand several hours, with frequent shaking, before the fractional distillation. Fractions boiling at from 73–78° may be used. A standardization curve is made for each fraction or batch of solvent.

*Hydrazine reagents.* Either 2,4-dinitrophenylhydrazine (Eastman, No. 1866) or the hydrochloride of 4-nitrophenylhydrazine (Eastman, No. 979) may be used. 2,4-Dinitrophenylhydrazine is preferred because it reacts more rapidly with keto acids, its solution is stable, and it may be used for the determination of "total hydrazones" as well as keto acids. 100 mg. are ground in a mortar with increasing small volumes of approximately 2 N HCl until 100 cc. have been added. The solution is filtered through a small filter paper and is then kept in the refrigerator when not in use.

*Sodium carbonate.* A 10 per cent solution.<sup>4</sup>

*Sodium hydroxide.* Solutions of approximately 1.5 N and 2.5 N strength are prepared.<sup>4</sup>

*Collection of Sample*—Blood is withdrawn from the vein by means of dry 2 or 5 cc. syringes fitted with 21 gauge hypodermic needles (20). It is withdrawn with a minimum of stasis before collection; the tourniquet is removed immediately after entry of the needle into the vein. This precaution should be observed, despite the fact that continued *moderate* stasis during the collection of the sample does not perceptibly affect the pyruvic and lactic acid levels. However, *the subject should be cautioned against clenching and opening the hand, since such muscular movements may affect the results.* Although the use of cold syringes is preferred, syringes cooled to the temperature of the room will yield satisfactory results. Warm syringes should never be used. The syringe is held vertically while the plunger is slowly moved upward to the mark.

The sample is then rapidly ejected in a fine stream through the needle into 5 volumes of cold solution of acid precipitant (trichloroacetic, metaphosphoric) contained in a cork-stoppered, 15 or 50 cc. centrifuge tube. The tube is then stoppered, shaken, and centrifuged, after which it is kept in the refrigerator until ready for analysis. Samples prepared in this manner have been stored 2 days in the cold without demonstrable loss of pyruvic acid or gain of lactic acid.<sup>5</sup> If lactic acid is to be determined,<sup>6</sup> a syringe of 5 cc. capacity is used; otherwise a tuberculin precision syringe of 2 cc. capacity is employed.

<sup>4</sup> If stored in ordinary bottles, the solutions soon contain considerable quantities of suspended material. They should be kept either in paraffin-lined bottles or in Pyrex containers. The carbonate solution should be filtered if it is not clear immediately after preparation.

<sup>5</sup> In the case of subjects in ketosis, the precipitated sample should stand overnight in the refrigerator to eliminate the slight effect of acetoacetic acid on the determination. The interfering effect of acetone in the determination of lactic acid is overcome by boiling the final filtrate from the Van Slyke  $\text{CuSO}_4\text{-Ca(OH)}_2$  reagents for a minute or two before the analysis is begun.

<sup>6</sup> If the Shaffer method (21) is used, the acid reagent should contain 50 cc. of syrupy (85 per cent) phosphoric acid and 200 gm. of manganese sulfate per liter.

24 hour specimens of urine are collected in large, cold bottles which contain 5 cc. of  $20 \times \text{H}_2\text{SO}_4$ . Single specimens are collected in cold containers, and 0.5 cc. of  $20 \times \text{H}_2\text{SO}_4$  is added for each 100 cc. of urine. At this acidity (0.1 to 0.05  $\times$ ), the pyruvic acid content of the urine is not changed appreciably during another 24 hours of storage in the refrigerator. 2 cc. of urine are added to 10 cc. of 10 per cent solution of metaphosphoric or trichloroacetic acid. This solution (when kept cold) will remain unchanged with respect to pyruvic acid for at least 24 hours.

Urine, or samples which contain indicators or other non-precipitable colored material, should be treated with Lloyd's reagent. 0.75 gm. of the reagent is added to each 10 cc. of strongly acidified urine or acid extract. A concentration of approximately 0.1  $\times$  acidity is recommended; at lower acidities appreciable quantities of keto acids may be lost. The mixture is filtered *immediately*.

#### *Extraction Method; General Procedure*

3 cc. of the cold, clear supernatant solution, or 3 cc. of the diluted sample of urine, are transferred to a small (18  $\times$  150 mm.) test-tube. If it is necessary to take a smaller sample, enough of the blank solution is added to bring the volume to 3 cc. The contents of the tube are warmed 10 minutes in a bath of water at approximately 25°. 1 cc. of the hydrazine reagent is then added, and the mixture is allowed to react for exactly 5 minutes.

Exactly 8 cc. of ethyl acetate or other suitable solvent<sup>7</sup> are now added and a rapid stream of nitrogen (or air) is passed through the mixture for a period of 2 minutes. Most of the lower aqueous layer is then removed by means of the Wright capillary pipette, through which the current of gas has passed. The test-tube is next given a sudden circular motion which dislodges the solution adhering to the walls. The remaining solution is then removed.

Exactly 6 cc. of sodium carbonate solution are now added to the solvent and a rapid current of gas is again passed through the mixture for a period of 2 minutes. The capillary tube is removed and the two phases are allowed to separate. A 5 cc. pipette is inserted quickly through the upper layer; air is momentarily blown through the pipette to discharge the aqueous extract and a small quantity of solvent. The solution is then drawn into the pipette and the tip of the pipette is wiped free of adhering solvent before the volume is adjusted to the mark. The contents are then transferred to a colorimeter tube.

<sup>7</sup> This is the largest volume of solvent which can be added without subsequent mechanical loss of the contents from the small test-tube during the aeration.



Exactly 5 cc. of 1.5 N NaOH solution are added and the contents of the tube are immediately mixed. Readings are made by means of the photo-electric colorimeter, with Filters 420 and 520, 5 to 10 minutes after addition of the alkali. The absorption of light by the colored hydrazone salt is determined after the apparatus is set to maximum transmission (100 galvanometer readings, or zero reference point, depending upon the type of colorimeter used) by means of the reagent blank.

When chloroform or ethyl ether is used, the tube is kept cold by placing it in a beaker containing ice water. This prevents loss of the solution by volatilization. If chloroform or other solvents which are heavier than water are used, the procedure must be changed as follows: After the first extraction, the upper layer is carefully removed by means of the Wright capillary pipette and discarded. The extraction of the lower chloroform layer with aqueous carbonate solution is then carried out as described above. 5 cc. of the upper layer are taken for the final development of color.

The presence of extractable indicators, dyes, or other colored materials may seriously affect the results. Should any such color remain after treatment with Lloyd's reagent, the error can be minimized by setting up two more tubes, C and D, as follows: Tube A, reagent blank + 1 cc. of hydrazine reagent; Tube B, sample + 1 cc. of hydrazine reagent; Tube C, reagent blank + 1 cc. of 2 N HCl; Tube D, sample + 1 cc. of 2 N HCl. The contents of Tubes A, B, C, and D are extracted as already described. The absorption of light by the soluble colored material in Tube D is determined after the apparatus is set to maximum transmission by means of the reagent blank, Tube C. The results are expressed as pyruvic acid; Tube B - D = corrected pyruvic acid content of the sample. Examples illustrating this method of correction for the color are given in Table II.

It should be noted that the method makes use of the distribution principle; therefore, *it is necessary to measure all solutions with care*. Because of the volatility of the solvents and other sources of error, *the determination should be carried to completion with a minimum of delay*. If many determinations are made, the hydrazine is added to successive samples at definite intervals (at, say, every 30 seconds). This permits accurate timing, which is essential in the analysis of urine. In this laboratory, seven determinations, including the blank, are made at one time, the time required being 45 minutes to 1 hour.

#### *Extraction Method; Specific Procedures for Pyruvic and Dicarboxylic Keto Acids*

Two sets of tubes with their appropriate blanks are set up as already described. 3 cc. of the protein-free trichloroacetic acid filtrate, or of the diluted sample in trichloroacetic acid solution, are warmed to 25°, after

which 1 cc. of 2,4-dinitrophenylhydrazine is added. The subsequent steps are shown in Table I.

Calibration curves are prepared by analyzing pyruvic acid standards by both procedures. All results are expressed in terms of pyruvic acid. *Procedure A is useful for the determination of total keto acids. Procedure B is highly specific for pyruvic acid; approximately 10 per cent of the  $\alpha$ -ketoglutaric acid and 5 per cent of oxalacetic acid can also be determined by it.* The results obtained by means of Filters 540, 420, and 400 permit the calculation of the approximate concentrations of each of the keto acids. The methods of calculation are given below.

TABLE I  
*Extraction Procedures A and B*

	Procedure A	Procedure B
Incubation of reaction mixture at $\pm 25^\circ$	25 min.	5 min.
Extraction; volume and solvent	8 cc. ethyl acetate or other non-specific solvent	3 cc. xylene, toluene, or benzene
Reextraction with carbonate solution	6 cc. 10% solution	6 cc. 10% solution
Alkali added to 5.0 cc. of extract	5.0 " 1.5 N NaOH	5.0 " 1.5 N NaOH
Light filter No.	540, 420, 400	520

*Direct Method; Total Hydrazones*

3 cc. of the cold protein-free blood extract are transferred to a colorimeter tube. The contents are warmed 10 minutes in a beaker of water at approximately  $25^\circ$ . 1.0 cc. of hydrazine reagent is then added. The mixture is now incubated 5 minutes, after which 5.0 cc. of 2.5 N NaOH are added. The color is determined 9 to 11 minutes after addition of the alkali. A reagent blank is prepared at the same time.

If the extract or diluted sample contains interfering colored material, four tubes (A, B, C, and D) are set up and the determination is carried out as described above. Examples showing the magnitude and the method of the correction for color in Tube D are given in Table II.

The effect of various substances on the determination is as follows: *less than 1 per cent*, hexoses, glucuronic acid, ascorbic acid, kojic acid; *1 to 10 per cent*, acetylpyruvic acid, acetoacetic acid, levulinic acid; *greater than 10 per cent*, formaldehyde (and urotropin), acetaldehyde, glyoxal, methylglyoxal, glyceraldehyde, acetone, glyoxylic acid, oxalacetic acid,  $\alpha$ -ketoglutaric acid.

## EXPERIMENTAL

*Preparation of Samples for Analysis*

*Precipitants*—The following protein precipitants were found unsatisfactory for the determination of pyruvic acid in blood: colloidal iron (22, 23), zinc (24), copper (25), and cadmium (26) hydroxides, and the Folin-Wu tungstic acid reagents (27).

Of all the precipitants so far used, trichloroacetic acid is the only one which has yielded almost quantitative recoveries, from 98 to 99 per cent of added keto acid. Its solutions, however, are unstable and, on long standing in a warm room, may contain considerable quantities of hydrazine-binding substances. Blood filtrates when allowed to stand 4 hours at 35–37° too may contain increased quantities of "pyruvic acid" equivalent to 0.05 to 0.15 mg. per 100 cc. of blood and considerably increased quantities, from 0.1 to 0.3 mg. per cent, of other reactive —CO— compounds. Under the same conditions, urine samples which have been diluted with 5 volumes of the reagent may contain from 0.1 to 0.6 mg. per cent more of "pyruvic acid" and slightly more of other reactive —CO— compounds.<sup>8</sup>

We suggest the use of metaphosphoric acid as a fairly satisfactory precipitant. The solutions are less stable than those of trichloroacetic acid, and blood filtrates are not as clear. The recovery of pyruvic acid added to samples of blood is not as great when metaphosphoric acid is used, from 92 to 94 per cent. However, the increase of hydrazine-binding substances in filtrates which are allowed to stand several hours in a warm room is consistently smaller than that in filtrates prepared with trichloroacetic acid.

The origin of these substances is not clear. The greater acidity of trichloroacetic acid filtrates, together with the elevated temperature, may bring about a slight increase of color by decomposition of hemoglobin and other substances; it favors dissociation of hexamethylenetetramine, which is always present in normal urine, and it accelerates the acetone decomposition of acetoacetic acid. These and other changes may account for the increase of total —CO— compounds, but not for the apparent increase of extractable chromogenic hydrazones, or keto acids. Some of the keto acid is probably derived from the decomposition of trichloroacetic acid, catalyzed by substances present in the samples.

*Lloyd's Reagent*—Many indicators and dyes are removed quantitatively

<sup>8</sup> In our experience, such conditions are by no means unusual. They may be encountered when samples are collected in trichloroacetic acid (20) during the warm months at some distance from the laboratory; for example, in studies carried out on the athletic field, the samples being brought to the laboratory some time later. Whenever inconsistent results have been obtained from such samples, it has been found that no precautions were taken to keep the precipitated samples cool.

by Lloyd's reagent in acid solution (0.1 N  $\text{H}_2\text{SO}_4$ ) as are, for example, methyl red, methyl orange, alizarin, litmus, fuchsin, eosin, saffranine, gentian violet (crystal violet), and methylene blue. The phthalein indicators are only partially removed. These indicators are readily extracted by the solvent and they are reextracted by the carbonate solution. They, therefore, interfere greatly in the determination (Standard 3, Table II). Lloyd's reagent removed approximately 85 per cent of the indicator. It did not perceptibly affect the recovery of pyruvic acid (Standards 2 and 4, Table II). 1 gm. of Lloyd's reagent for each 10 cc. of solution is perhaps

TABLE II

*Lloyd's Reagent; Removal of Color and Recovery of Added Pyruvic Acid*

The density of the color was determined by means of the Evelyn colorimeter, with Filter 520. The analytical data are expressed as mg. of pyruvic acid per 100 cc.

Sample	Pyruvic acid added	0.04 per cent phenol red	Lloyd's reagent	Direct method			Extraction method, ethyl acetate solvent		
				Tube B	Tube D	Tube B - D	Tube B	Tube D	Tube B - D
	mg. per 100 cc.	cc. per 100 cc.	gm. per 10 cc.						
Standard 1	2.38	None	None	2.38	0	2.38	2.38	0	2.38
" 2	2.38	"	1.0	2.35	0	2.35	2.35	0	2.35
" 3	2.38	5.0	None	3.84	1.43	2.41	3.89	1.42	2.47
" 4	2.38	5.0	1.0	2.53	0.18	2.35	2.58	0.21	2.37
" 5	2.38	5.0	3.0	2.05	0.16	1.89	1.91	0.21	1.70
Urine 1a			None	3.50	0.28	3.22	2.46	0.14	2.32
" 1b			1.0				2.24	0.12	2.12
" 2a			None	3.41	0.34	3.07	2.97	0.20	2.77
" 2b			1.0				2.68	0.11	2.58
" 3a			None	3.69	0.40	3.29	3.02	0.17	2.85
" 3b			1.0				2.57	0.02	2.55
" 3c	2.19		None				4.95	0.08	4.87
" 3d	2.19		1.0				4.67	0.02	4.65

the maximum which can be added without loss of pyruvic acid. Thus, the addition of 3 gm. of Lloyd's reagent to 10 cc. of Standard 5 led to a marked loss of keto acid and no further removal of the indicator.

The acidity of the solution is also important. Thus, only 81 per cent of pyruvic acid was recovered from a standard (not shown in Table II) which contained only 5 drops of N HCl per 100 cc. In all of these determinations the filtration was begun within a minute or two after the reagent was added and mixed.

Urine was collected from normal human subjects over a period of 24 hours. Three samples whose volumes were less than 1200 cc. and which

were somewhat more deeply colored than usual were taken for analysis. The results in Table II show that about one-half of the urinary pigment was removed by treatment with Lloyd's reagent.

A satisfactory recovery of added pyruvic acid was obtained, provided not more than 1 gm. of Lloyd's reagent was added to 10 cc. of urine. 5 cc. of water, or standard solution which contained 2.19 mg. of pyruvic acid, were added to 100 cc. of Urine 3. The increase of pyruvic acid in the untreated sample Urine 3c was 4.87 - 2.85, or 2.02 mg. per cent. The increase of pyruvic acid in Urine 3d, which was treated with Lloyd's reagent, was 4.65 - 2.55, or 2.10 mg. per cent.

TABLE III

*Lloyd's Reagent; Recovery of  $\alpha$ -Ketoglutaric and Oxalacetic Acids*

3.0 cc. of sample in trichloroacetic acid were incubated 20 minutes with 2,4-dinitrophenylhydrazine to insure complete reaction of all keto acids. The extractions were carried out with ethyl acetate and 10 per cent  $\text{Na}_2\text{CO}_3$  solution. The Evelyn colorimeter, Filter 520, was used. The results are expressed as mg. per cent of pyruvic acid.

Keto acid added	Standard		Urine	
	Untreated	Lloyd's reagent, 1.0 gm. per 10 cc. sample	Untreated	Lloyd's reagent, 1.0 gm. per 10 cc. sample
	mg. per cent	mg. per cent	mg. per cent increase	mg. per cent increase
$\alpha$ -Ketoglutaric.....	1.00	1.00	1.04	0.92
Oxalacetic.....	1.06	1.04	1.00	1.03
Pyruvic.....	1.09	1.09	1.00	1.00

Similar satisfactory recoveries were obtained when equivalent quantities of  $\alpha$ -ketoglutaric and oxalacetic acids were added to urine. See Table III.

Although Lloyd's reagent thus does not remove appreciable quantities of added keto acids, it does remove substances whose hydrazones are extracted by the solvent and which therefore interfere in the determination of pyruvic acid. Thus, Urines 1b, 2b, and 3b of Table II, which were treated with the earth, contained 0.20, 0.19, and 0.30 mg. per cent less of "pyruvic acid" than the untreated samples. Since this procedure increases the specificity of the method for  $\alpha$ -keto acid, we recommend that all samples of urine be treated with Lloyd's reagent.

*Comparison of Authors' Method with That of Lu, Bueding, and Wortis*

Before a discussion of the reaction of carbonyl compounds with the nitrophenylhydrazines and the factors which influence the extraction of the

resulting hydrazones, it should be shown that the authors' *single extraction* procedure and the procedures of Lu, Bueding, and Wortis, which depend upon *complete extraction*, yield identical results. This can be seen in Table IV. In these determinations separate standardization curves were prepared for each of the procedures, including the authors' modified procedure. In the latter, the carbonate extracts were allowed to stand 1

TABLE IV  
*Comparison of Results Obtained with Lu, Bueding, and Wortis Method and Authors' General Method, Ethyl Acetate As Solvent*

Standardization curves were prepared for each of the procedures, including the authors' modified procedure.

Sample	Pyruvic acid		$\frac{L_{410}}{L_{415}}$		Pyruvic acid, modified authors' method*
	Lu, Bueding, and Wortis method	Authors' method	Lu, Bueding, and Wortis method	Authors' method	
	mg. per cent	mg. per cent			
Blood 1	3.13	3.30	1.32	1.34	3.14
" 2	1.30	1.43	1.36	1.29	1.30
" 3	0.65	0.64	1.30	1.34	0.67
" 4	1.04	1.04	1.37	1.27	1.08
" 5	0.84	0.86	1.40	1.32	0.86
" 6	1.24	1.22	1.32	1.33	1.24
" 7	0.93	0.95	1.37	1.34	0.94
" 8	0.70	0.73	1.43	1.39	0.73
Urine 1	1.22	1.17	1.48	1.41	1.16
" 2	1.28	1.25	1.49	1.50	1.26
" 3	1.86	1.94	1.52	1.41	
" 4	1.96	2.16	1.55	1.41	
" 5	1.02	1.11	1.47	1.32	
" 6	2.28	2.45	1.51	1.40	
" 7	1.62	1.92	1.78	1.56	
" 8	1.32	1.32	1.51	1.49	1.34
" 9	0.47	0.48	1.63	1.52	0.60
" 10	2.80	2.91	1.50	1.43	2.80

\* The sodium carbonate extract was allowed to stand 1 hour at room temperature before the alkali was added; see the text.

hour exposed to air at room temperature before the alkali was added and the final determination was made. This appeared to be necessary because the results obtained by the authors' method in many instances were higher than those obtained by the Lu, Bueding, and Wortis method. Apparently the carbonate extracts from urine and blood contain substances which, during the long time required to make the last four extractions, bring about the oxidation of pyruvic acid. When the authors' method was modified

to make the conditions approximately the same in this respect, the two methods yielded results which agreed within the limits of experimental error.

*Specificity*—Case, Peters and Thompson, and Lu have shown that their extraction procedures are specific for keto acids. The degree of specificity under the conditions of our method is summarized in Table V. It will be seen that the  $\alpha$ -keto acids are determined equally well. On the other hand, by this procedure only about 6 per cent of acetoacetic acid, which is

TABLE V

*Reaction of Carbonyl Compounds with 2,4-Dinitrophenylhydrazine and Properties of Hydrazones*

Substance		Time necessary for complete reaction at 25°	Authors' extraction method				Authors' direct method
			Interference in determination of pyruvic acid* (Filter 520)	Hydrazone extracted by 8 cc. solvent		Light absorption of hydrazones in alkali*	Light absorption of hydrazones in alkali
				Ethyl acetate	Benzene		
		min.	per cent	per cent	per cent	$\frac{L_{420}}{L_{520}}$	$\frac{L_{420}}{L_{520}}$
$\alpha$ -Keto acids	Pyruvic	>5	100	81	93	1.28-1.33	1.28-1.40
	Oxalacetic	20-25	100	79	19	1.28-1.32	1.28-1.32
	$\alpha$ -Ketoglutaric	20-25	100	89	33	1.95-2.00	1.55-1.65
$\beta$ -Keto acids	Acetoacetic	>5	6			1.40-1.45	1.35-1.40
$\gamma$ -Keto acids	Levulinic	>5	1			1.30-1.35	1.40-1.45
Aldehydes	Glyceraldehyde	>5	>1				1.60-1.70
	Acetaldehyde	>5	>1				2.85-2.90
	Formaldehyde	>5	>1				7.10-8.10
Ketones	Acetone	>5	>1				1.45-1.50
Glyoxals	Methylglyoxal	>5	>1				0.45-0.50
	Glyoxal	>5	>1				0.30-0.35

\* Ethyl acetate solvent.

a  $\beta$ -keto acid, is determined, and approximately 1 per cent of levulinic acid, which is a  $\gamma$ -keto acid. The method, therefore, is most specific for the  $\alpha$ -keto acids.

#### *Reaction of Keto Acids with Phenylhydrazines*

*Rate of Reaction*—Equivalent quantities of pyruvic and  $\alpha$ -ketoglutaric acids (14.5 and 24.1  $\gamma$ , respectively, in 3 cc. of trichloroacetic acid solution) were allowed to react with various nitrophenylhydrazines at 25°. After a specified period of time, 8 cc. of ethyl acetate were added and the extrac-

tions were carried out immediately. The color of the alkali hydrazones was measured in the Evelyn colorimeter, with Filter 520.<sup>9</sup> The results are shown in Fig. 1. Results are not shown for 3-nitrophenyl- and 2,4,6-trinitrophenylhydrazines, since no apparent increase of color was noted even after 1 hour of incubation.

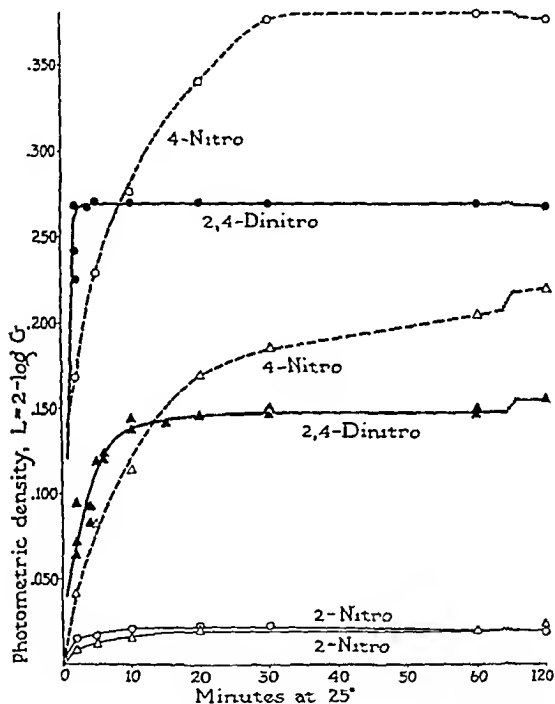


FIG. 1. Rate of reaction of pyruvic acid (O) and  $\alpha$ -ketoglutaric acid ( $\Delta$ ) with nitrophenylhydrazines. Ethyl acetate solvent was used, and the readings were taken with Filter 520.

2-Nitrophenylhydrazine itself yields a pale yellow color on the addition of alkali. Its keto acid hydrazones in alkali also are yellow in color. The rate of reaction of this hydrazine with keto acids was approximately the same as that of 2,4-dinitrophenylhydrazine. On the other hand, 4-nitrophenylhydrazine reacted very slowly. The reaction of the 4-nitro com-

<sup>9</sup> The light filters were obtained from the Rubicon Company, Philadelphia, Pennsylvania, the makers of the Evelyn colorimeter.



pound with pyruvic acid was complete in about 30 minutes; the reaction with  $\alpha$ -ketoglutaric acid required 120 minutes for completion.

The reaction of 2,4-dinitrophenylhydrazine with pyruvic acid was complete in 3 to 4 minutes at 25°. The following compounds also gave maximum results within less than 5 minutes of incubation at 25°: formaldehyde, acetaldehyde, glyoxal, glyoxylic acid, glyceraldehyde, methyl-

TABLE VI

*Rate of Reaction of Keto Acids of Blood and Urine with 2,4-Dinitrophenylhydrazine at 25°*

All urine samples were treated with Lloyd's reagent. The extractions were made with c.p. ethyl acetate. The results are expressed in mg. per cent.

Sample	Extractable hydrazones, "pyruvic acid"			Total hydrazones (corrected) expressed as pyruvic acid			Extraction method after 5 min.
	After 5 min.	After 20 min.	After 60 min.	After 5 min.	After 20 min.	After 60 min.	
Blood A1*	1.17	1.16	1.19	1.37	1.53	1.60	$\frac{L_{20}}{L_{10}}$ 1.32
" A2*	1.16	1.16	1.16	1.34	1.50	1.68	1.38
" B	0.76	0.77	0.77	0.87	1.13	1.20	1.38
" C	1.62	1.62	1.64	2.01	2.23	2.30	1.30
Urine A	1.12	1.54	1.68	1.64	2.32	2.95	1.47
" B	0.52	0.72	0.78	0.73	1.02	1.54	1.42
" C	2.21	2.98	3.33	3.06	4.18	4.17	1.50
" D	1.27	1.61	1.67	1.53	1.98	2.59	1.48
" E <sub>1</sub> †	0.14	0.24	0.26	0.25			1.65
" E <sub>2</sub>	10.6	11.1	10.9	11.3	11.8	12.8	1.31
" E <sub>3</sub>	23.0	23.3	23.2	24.5	25.1	26.5	1.28
" E <sub>4</sub>	18.8	19.2	19.4	21.0	21.9	22.3	1.30
" E <sub>5</sub>	3.58	3.91	3.93	3.99	4.25	4.58	1.37

\* Blood A1 was taken at 9.45 a.m.; Blood A2 5 minutes after the subject had walked 600 feet.

† The subject ran as rapidly as possible up seventeen flights of steps. In order to increase the flow of urine he drank much water during the 2 hours preceding and the 1st hour following the period of exercise. Urine E<sub>1</sub> was collected just before the experiment; Urines E<sub>2</sub> to E<sub>5</sub> at 15 minute intervals after the exercise.

glyoxal, pyruvic acid, acetoacetic acid, acetone, and levulinic acid. *Oxalacetic and  $\alpha$ -ketoglutaric acids required approximately 20 minutes for complete reaction.*

The reaction of hydrazine with the carbonyl compound apparently proceeds only in the aqueous acid solution and does not continue in the solvent phase, as is shown by the following experiment.  $\alpha$ -Ketoglutaric acid solutions were allowed to react exactly 2 minutes at 25° with 2,4-dinitrophenyl-

hydrazine, after which the reaction mixture was extracted immediately with ethyl acetate. Immediate reextraction of the solvent phase with carbonate yielded 45, 47, and 53 per cent of the maximum color; reextraction after 30 minutes yielded 46, 49, and 40 per cent of the maximum color. These data demonstrate, first, that it is possible to stop the reaction at any time merely by extracting the mixture with a solvent and immediately

TABLE VII

*Extraction of Keto Acid Hydrazones and 2,4-Dinitrophenylhydrazine by Various Solvents*

Approximately equimolecular quantities of the keto acids were allowed to react to completion with 1 cc. of 2,4-dinitrophenylhydrazine solution at 25°. The reaction mixture was then extracted with 8 cc. of the solvent indicated in the first column.

Solvent	Pyruvic acid hydrazone		$\alpha$ -Ketoglutaric acid hydrazone		Oxalacetic acid hydrazone		2,4-Dinitrophenylhydrazine	
	Carbonate extract	Per cent extracted	Carbonate extract	Per cent extracted	Carbonate extract	Per cent extracted	Residual color of reaction mixture	Per cent extracted
	$L_{520}$		$L_{520}$		$L_{520}$		$L_{520}$	
None.....	0.335*		0.312*		0.322*		0.599†	
Benzene.....	0.312	93	0.101	33	0.061	19	0.350	42
Toluene.....	0.310	93	0.091	29	0.057	18	0.262	56
Xylene.....	0.317	95	0.084	27	0.057	18	0.319	47
Chloroform.....	0.318	95	0.215	69	0.101	31	0.191	68
Ethyl ether.....	0.308	92	0.288	92	0.300	93	0.288	52
Caprylic alcohol....	0.285	85	0.273	88	0.305	95	0.039	93
Ethyl acetate, c.p.....	0.271	81	0.245	79	0.286	89	0.008	99
" " technical..	0.241	72	0.214	69			0.007	99

\* By direct determination, without previous extraction. The other results in the column were obtained by multiplying the observed density,  $L_{520}$ , by 6/5, since 5 cc. of the 6 cc. of carbonate extract were taken for development of color.

† A mixture, consisting of 3.0 cc. of trichloroacetic acid solution and 1.0 cc. of hydrazine solution, was extracted with 8.0 cc. of solvent. The aqueous layer was then quantitatively transferred to a colorimeter tube; 6.0 cc. of water were added, and the yellow color was determined, with Filter 420.

thereafter removing the aqueous phase, and, second, that the determination can then be carried out more leisurely after this extraction.

*Accurate timing is not so necessary in the analysis of the blood, but it is of the greatest importance in the analysis of urine or materials which contain dicarboxylic keto acids and other slowly reacting substances. This is shown by the results in Table VI.*

#### *Absorption of Light by Alkali Hydrazones*

12.27  $\gamma$  of pyruvic acid and equivalent quantities of oxalacetic or  $\alpha$ -ketoglutaric acids in 3 cc. of trichloroacetic acid solution were incubated with

various hydrazines at 25° until the reaction was complete. The extractions and the development of color were then carried out as usual, and the photometric density was determined by means of the Evelyn colorimeter. Ethyl acetate solvent was used because it is most frequently used by other workers and because it is one of the few solvents which extracts approximately equal quantities of the keto acid hydrazones (Table VII).

It should be pointed out that the curves given in Fig. 2 are not identical with those given by equivalent quantities of the pure hydrazones. The keto acid hydrazones exhibit marked differences in solubility (see Table VII), and the final solution therefore may not contain exactly equivalent

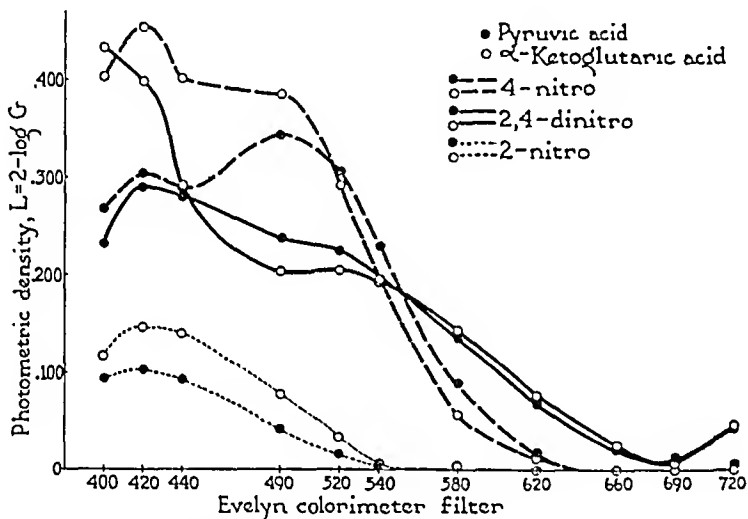


FIG. 2. Light absorption of nitrophenylhydrazones of pyruvic and  $\alpha$ -ketoglutaric acids. Ethyl acetate solvent was used.

lent quantities of the hydrazones, even though the reaction mixture in every case contained an equivalent quantity of keto acid. The final solution also contains varying quantities of other impurities, which may affect the absorption of light. Fig. 2 therefore contains comparative results, such as are obtained under the conditions of the authors' procedure.

The hydrazones which resulted from the reaction of the three nitrophenylhydrazines differed greatly in their light-absorbing properties in strongly alkaline solution. These results indicate that the red color of the 2,4-dinitrophenylhydrazones in strong alkali is probably due to substitution of one of the nitro groups in the para position of the benzene ring.

Of particular interest is the similarity of the curves of the pyruvic and oxalacetic acid hydrazones (Fig. 3). The curves of all three keto acids

coincided at wave-lengths greater than 540 m $\mu$ . The curve for  $\alpha$ -keto-glutaric acid hydrazone differed markedly from that of the other keto acids at lower wave-lengths.

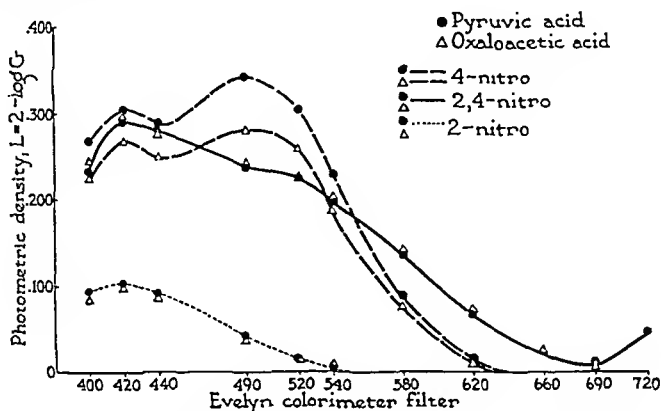


FIG. 3. Light absorption of nitrophenylhydrazones of pyruvic and oxalacetic acids. Ethyl acetate solvent was used.

TABLE VIII

*Ratio of  $L_{420}$ : $L_{520}$ ; Analysis of Solutions Containing Varying Quantities of Keto Acids*

5.0 cc. of the solution, whose composition is shown in the table, were added to 25 cc. of trichloroacetic acid solution. 3.0 cc. volumes were incubated at 25° with 1 cc. of 2,4-dinitrophenylhydrazine. Single extractions were made with 8 cc. of solvent and 6 cc. of 10 per cent sodium carbonate solution.

Solution			c.p. ethyl acetate solvent	
Pyruvic acid	$\alpha$ -Ketoglutaric acid	Molecular ratio of pyruvic to $\alpha$ -keto-glutaric acid	5 min. incubation	20 min. incubation
mg. per cent	mg. per cent		$\frac{L_{420}}{L_{520}}$	$\frac{L_{420}}{L_{520}}$
2.454	0	100:0	1.27	1.29
2.209	0.4064	90:10	1.32	1.38
1.963	0.8128	80:20	1.38	1.44
1.718	1.2192	70:30	1.44	1.50
1.227	2.032	50:50	1.56	1.64
0.6135	3.048	25:75	1.73	1.81
0	4.064	0:100	1.97	1.99

*Identification of Keto Acids by Means of Ratio  $L_{420}$ : $L_{520}$*

The differences in the absorption of light by various hydrazones may be characterized by the ratio of the photometric densities at two wave-lengths

of light, say at 420 and 520  $m\mu$ . This is shown in Table V. Each substance yielded ratios which varied somewhat, depending upon the concentration and the solvent used in the first extraction. It was approximately the same whether determined directly or after extraction. The glyoxals yielded the lowest ratios. This was to be expected, since the alkali hydrazones of glyoxals have a violet color (1-3). The highest ratios were obtained from the paraffin aldehydes, formaldehyde and acetaldehyde.

Since the extraction method is specific for keto acids, especially for the  $\alpha$ -keto acids, and since the  $L_{420}:L_{520}$  ratio yielded by one of these acids is markedly higher than that of the other two, this property may be used not only to identify the acids but also to determine the relative quantity of each. Thus, a ratio considerably greater than 1.30 indicates the presence of  $\alpha$ -ketoglutaric acid. If the sample contains  $\alpha$ -ketoglutaric acid, and relatively few other substances, the ratio can be used to calculate the relative quantity of  $\alpha$ -ketoglutaric and pyruvic acids. A marked increase of the ratio is observed when  $\alpha$ -ketoglutaric acid constitutes as little as 10 per cent of the total keto acids, as can be seen in the last column of Table VIII. The quantitative application of this principle is shown in Table XIII.

### *Solvents*

Many solvents have been used in the past. The early workers extracted the crystalline mass of hydrazones with 10 per cent sodium carbonate (1, 5) or dilute ammonia (6) solution; the hydrazones, after recovery from the alkaline solution, were crystallized from nitrobenzene (1) or ethyl acetate (7). Case and Peters and Thompson extracted the aqueous reaction mixture with ethyl acetate; the extract was evaporated, the residue was redissolved in toluene, and the toluene solution was finally extracted with sodium carbonate solution. Lu and others (14, 16) extracted the reaction mixture with ethyl acetate. Pi-Suñer and Farrán used toluene, and Larsson and Liljedahl used ethyl ether.

*Use of Various Solvents*—The solvents listed in Table VII were the best of many tested. They represent many types of compounds: esters, alcohols, ethers, chlorinated hydrocarbons, and aromatic hydrocarbons. They are arranged approximately in the order of their efficiency in extracting the pyruvic acid hydrazone from the trichloroacetic acid reaction mixture. The aromatic hydrocarbons, chloroform, and ether extracted from 92 to 95 per cent of pyruvic acid hydrazone. Caprylic alcohol extracted somewhat less, and ethyl acetate the least. Turpentine, which contains terpenes similar in structure to the aromatic hydrocarbons, and kerosene, which consists of a mixture of lower paraffin hydrocarbons, proved unsatisfactory for the extraction of hydrazones. Likewise, carbon tetrachloride and butyl, isobutyl, and amyl alcohols were found unsuitable.

The results in Table VII demonstrate a marked difference in the degree of solubility of the hydrazones of the dicarboxylic acids in the various solvents. Approximately 30 per cent of the  $\alpha$ -ketoglutaric and 20 per cent of the oxalacetic acid hydrazones were extracted by the aromatic hydrocarbons. These hydrocarbons also extracted much less of the unused dinitrophenylhydrazine. Since they extracted more than 90 per cent of the pyruvic acid hydrazone, their use is especially recommended for the determination of pyruvic acid. Ethyl ether, caprylic alcohol, and c.p.

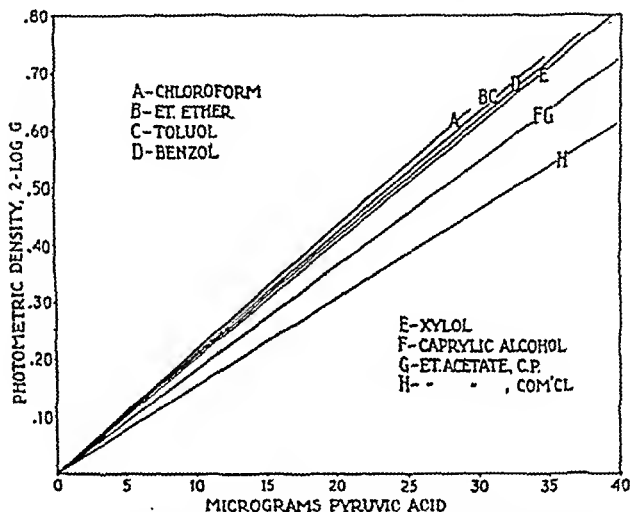


FIG. 4. Standardization curves obtained with various solvents. Pyruvic acid standards were allowed to react 5 minutes with 2,4-dinitrophenylhydrazine, after which the extraction was carried out with 8 cc. of solvent. Filter 520 was used throughout.

ethyl acetate extracted approximately equal quantities of the three hydrazones.

When pyruvate standards were analyzed (Fig. 4), each of the solvents yielded results which followed Beer's law; the photometric density was proportional to the quantity of keto acid analyzed. The results obtained with ethyl acetate are of particular interest. This solvent, unlike the others, is difficult to prepare pure or to obtain in the anhydrous state. Alcohol, which is a common impurity and which has practically the same boiling point, is removed with difficulty. Alcohol, water, and the varying quantities of denaturant, methylisobutyl ketone, which are present in all

grades of ethyl acetate, have a decided effect on the extraction of the various hydrazones. It is therefore not the best solvent, although it is perhaps the least toxic.

TABLE IX

*Comparison of Results Obtained with Various Solvents*

All urine samples were treated with Lloyd's reagent. The density was determined with Filter 520. The results are expressed as mg. of pyruvic acid per 100 cc. of blood or urine.

Sample	Solvent					
	Ethyl acetate	Benzene	Toluene	Xylene	Chloroform	Caprylic alcohol
Blood 1	1.54	1.66			1.61	
" 2	0.72	0.94			0.95	
" 3	0.77	0.77		0.78		
" 4	1.13	1.27		1.30		
" 5	1.19	1.34		1.42		
" 6	2.75		3.19			2.56
" 7	0.99		1.20			0.82
" 8	1.70		2.01			1.62
" 9	0.62		0.86	0.80	0.80	
" 10	1.0		1.16	1.11	1.1	
Urine 1	1.47	1.30			1.32	1.53
" 2	0.51	0.50			0.46	0.49
" 3	3.80		2.48	2.56		4.20
" 4	3.20		1.66	1.65	2.31	
" 5	1.44		0.92	0.97	1.19	
" 6	2.06	1.13	0.98		1.59	
" 7	2.83		1.95	1.90	1.96	

TABLE X

*Ratio of  $L_{420}:L_{520}$ , Obtained in Analysis of Blood and Urine Samples Shown in Table IX*

Solvent	Blood 6	Blood 7	Blood 8	Blood 9	Blood 10	Urine 1	Urine 2	Urine 3	Urine 4	Urine 5	Urine 6	Urine 7
Ethyl acetate..	1.32	1.39	1.34	1.41	1.27	1.51	1.41	1.68	1.58	1.59	1.53	1.50
Benzene.....						1.37	1.29				1.36	
Toluene.....	1.33	1.35	1.33	1.41	1.29			1.42	1.33	1.34	1.32	1.29
Xylene.....				1.32	1.32			1.40	1.33	1.36		1.31
Chloroform....				1.37	1.35	1.39	1.41		1.47	1.51	1.42	1.37
Caprylic alcohol.....	1.32	1.35	1.32			1.51	1.40	1.59				

Marked differences were noted when these solvents were used in the analysis of urine (Table IX). Such differences were to be expected, since urine contains considerable quantities of  $\alpha$ -ketoglutaric acid. Caprylic alcohol and ethyl acetate gave the largest yields of "pyruvic acid." The

aromatic solvents, on the other hand, gave the smallest yields. These results are in agreement with the trends shown in Table VII.

When blood was analyzed, the aromatic hydrocarbons gave slightly higher yields of pyruvic acid. The differences were greater than the analytical error (see also Analyses 3 and 4 with Filter 520, Table XII). On the whole, however, the results obtained with the various solvents agreed fairly well.

The effect of the solvent on the ratio  $L_{420}:L_{520}$  is shown in Table X. When blood was analyzed, the various solvents yielded almost identical ratios. When urine was analyzed, ratios which approached those obtained

TABLE XI  
*Effect of Volume of Solvent*

The second extraction was made with a 5 per cent solution of sodium carbonate.

Keto acid*	Volume of solvent	Benzene		Ethyl acetate	
		$L_{420}$	Increase or decrease (3 cc. = 100 per cent)	$L_{420}$	Increase or decrease (3 cc. = 100 per cent)
	cc.		per cent		per cent
Pyruvic	3	0.258	100	0.244	100
"	5	0.268	104	0.237	97
"	7	0.273	106	0.232	95
"	10	0.268	104	0.226	93
$\alpha$ -Ketoglutaric	3	0.046	100	0.177	100
"	5	0.065	141	0.167	94
"	7	0.082	178	0.162	92
"	10	0.101	220	0.164	93

\* Equimolecular quantities were allowed to react to completion with 2,4-dinitrophenylhydrazine, after which the reaction mixture was extracted once with the volume of solvent indicated in the second column.

with pure pyruvic acid were obtained only when the aromatic solvents were used.

*Effect of Volume of Solvent*—That the extraction of pyruvic acid hydrazone is relatively little affected by the volume of solvent is shown in Table XI. As the volume of benzene used for the extraction was increased, the photometric density of the final solution was increased slightly; the reverse was noted when ethyl acetate was used. Proportionate slight decreases were seen when the  $\alpha$ -ketoglutaric hydrazone was extracted by increasing volumes of the ester. On the other hand, more than twice as much (220 per cent) hydrazone was extracted by 10 cc. of benzene as by 3 cc. From a consideration of the data in Table VII it is evident that the extraction procedure can be made more specific for pyruvic acid by using a small volume of an aromatic hydrocarbon.



### Summary of Conditions Which Affect Determination of Keto Acids

In the foregoing discussion it was shown that the following factors markedly affect the specificity: (1) the period of incubation, (2) the choice of light filter, (3) the solvent, and (4) the volume of solvent used in the extraction. The effect of these conditions on the analysis of an equi-

TABLE XII  
Summary; Effect of Various Factors on Specificity of Extraction Method

Analy- sis No.	Analytical conditions				Pyruvic + $\alpha$ -keto- glutaric acid stand- ard†	Urine (uncor- rected)†	Blood†	$\frac{L_{420}}{L_{540}}$		
	Incubation time	Light filter No.	Solvent	Vol- ume of solvent				Pyru- vic + $\alpha$ -keto- glutaric acid stand- ard	Urine	Blood
	min.			cc.	mg. per cent pyruvic acid	mg. per cent pyruvic acid	mg. per cent pyruvic acid			
1	60	420	Ethyl acetate	8	5.36	6.18	2.88	1.65	1.47	1.40
2	20	420	" "	8	<b>5.18</b>	<b>5.90</b>	<b>2.78</b>	1.61	1.49	1.36
3	5	420	" "	8	4.60	4.91	2.62	1.58	1.48	1.32
3	5	520	" "	8	3.78	4.31	2.57	1.58	1.48	1.32
4	5	520	Xylene	8	2.68	3.12	2.80	1.38	1.33	1.31
5	5	520	"	3	<b>2.41*</b>	<b>3.18</b>	<b>2.78</b>	1.38	1.32	1.33
6	5	520	"	3	2.44	3.26	2.84			
3	5	540	Ethyl acetate	8	3.88	4.29	2.51			
2	20	540	" "	8	<b>4.35*</b>	<b>5.06</b>	<b>2.67</b>			
1	60	540	" "	8	1.38	5.41	2.70			

\* The standard solution contained 2.21 mg. per cent of pyruvic acid and an equivalent quantity, 3.69 mg. per cent, of  $\alpha$ -ketoglutaric acid. The total keto acid content, expressed as pyruvic acid, should therefore be 4.42 mg. per cent.

† The bold-faced figures represent the results obtained by Procedures A and B, described in the text.

‡  $L_{420}:L_{520}$  ratios for pure pyruvic acid in this experiment were as follows: with ethyl acetate solvent, 1.31, 1.30, 1.31; with xylene solvent, 1.33, 1.32, 1.29. The  $L_{420}:L_{540}$  ratios were, with ethyl acetate solvent, 1.46, 1.46, 1.46; with xylene solvent, 1.49, 1.48, 1.47.  $\alpha$ -Ketoglutaric acid yielded the following ratios: with ethyl acetate solvent,  $L_{420}:L_{520}$ , 1.99, 2.00, 1.95;  $L_{420}:L_{540}$ , 2.08, 2.05.

molecular mixture of keto acids, urine, and blood is shown in Table XII. In each of the six series of analyses representing a total of ten conditions, pyruvic acid standards were simultaneously analyzed. In each case, the results were calculated by referring to the appropriate standardization curve. It will be seen (Analyses 1, 2, and 3) that the reaction was almost complete within 20 minutes, and that the lowest results were obtained when the period of incubation was only 5 minutes. The effect of the vari-

ous light filters on the results is shown by Analysis 3. The striking differences observed with pure solutions of keto acids when a non-specific solvent such as ethyl acetate and a more specific solvent such as one of the aromatic hydrocarbons (see Table VII) was used is shown by Analyses 3 and 4. The effect of the use of a small volume of a specific solvent (Table XI) is shown by Analyses 4 and 5.

As the conditions changed in Analyses 1 to 5, the determination became increasingly more specific for pyruvic acid. This was shown also by the ratios of  $L_{420}:L_{520}$ , which diminished proportionately. In addition to the 2.21 mg. per cent of pyruvic acid in the equimolecular mixture of keto acids, 10 per cent of the  $\alpha$ -ketoglutaric acid was determined in Analysis 5; the  $L_{420}:L_{520}$  ratio indicated the analysis of 11 per cent of the dicarboxylic acid (see foot-note, Table XII). Analysis 2 (with Filter 540) represented the least specific condition, and therefore in it the total keto acids should have been determined. Analysis of the mixed standard by this procedure indicated a recovery of 97 per cent of  $\alpha$ -ketoglutaric acid.

#### *Determination of Individual Keto Acids by Extraction Procedures A and B*

By carrying out determinations by Procedures A and B, it is possible to calculate the approximate content of the various keto acids. The calculation can be made directly from the  $L$  values, but it is simpler to express all results in terms of pyruvic acid and to base the calculations on the latter. Since oxalacetic acid is slowly decomposed by the acid precipitants (yielding pyruvic acid) and since it is present in relatively small concentrations, if at all, in body fluids and tissues, its effect is not apparent under the usual conditions. It can, however, be determined with considerable accuracy if the sample is precipitated (or diluted) with *cold* acid precipitant and the analysis carried out with a minimum of delay. However, since the analysis is usually delayed, the sample for all practical purposes contains only two keto acids, pyruvic and  $\alpha$ -ketoglutaric. This greatly simplifies the calculations.

*Method 1*—Let  $A_1$ ,  $A_2$ , and  $A_3$  represent the apparent keto acid content, determined by means of Procedure A, with Filters 540, 420, and 400, respectively. Let  $B$  represent the apparent keto acid content, determined by means of Procedure B. Finally, let  $p$  and  $g$  represent pyruvic and  $\alpha$ -ketoglutaric acids, respectively.  $A_1$ ,  $A_2$ ,  $A_3$ ,  $B$ ,  $p$ , and  $g$  are all expressed in terms of mg. per cent of pyruvic acid. On the basis of the results given in Tables XII and XIII<sup>10</sup>

<sup>10</sup> Equations 1 to 4 will differ somewhat in each laboratory, owing to slight differences of technique, of light filters, and of composition of solvents. These equations are given here to illustrate the method of calculation.

- (1)  $B = p + 0.10g$   
 (2)  $A_1 = p + 0.95g$   
 (3)  $A_2 = p + 2.06/1.46 (0.98g)$   
 (4)  $A_3 = p + 2.21/1.19 (0.98g)$

By subtracting Equation 1 from Equation 2,  $g = (A_1 - B)/0.85$ . Since  $\alpha$ -ketoglutaric acid =  $146g/88$ ,

- (5)  $\alpha$ -Ketoglutaric acid =  $1.95 (A_1 - B)$

TABLE XIII

*Separate Determination of Keto Acids in Mixtures by Specific Extraction Procedures A and B*

Pyruvic acid,  $L_{420}:L_{540}$  1.49,  $L_{400}:L_{540}$  1.19;  $\alpha$ -ketoglutaric acid,  $L_{420}:L_{540}$  2.11,  $L_{400}:L_{540}$  2.21.

The results are expressed in mg. per cent.

Solution			"Pyruvic acid," based on standardization of each procedure against pure pyruvic acid				Observed by Procedure A $\frac{L_{420}}{L_{400}}$	Method 1		Method 2*	
Molecular ratio of pyruvic to $\alpha$ -ketoglutaric acid	Pyruvic acid	$\alpha$ -Ketoglutaric acid	Procedure A			Procedure B Filter 520, B		Pyruvic acid by Equation 6	$\alpha$ -Ketoglutaric acid by Equation 5	Pyruvic acid by Equation 11	$\alpha$ -Ketoglutaric acid by Equation 12
			Filter 540, A <sub>1</sub>	Filter 420, A <sub>2</sub>	Filter 400, A <sub>3</sub>						
100:0	2.940	0	2.94	2.94	2.94	2.94	1.49	2.94	0		
83.3:16.7	2.45	0.82	2.95	3.12	3.34	2.48	1.57	2.42	0.93	2.57	0.66
66.7:33.3	1.96	1.63	2.90	3.29	3.80	2.03	1.69	1.92	1.72	1.90	1.63
33.3:66.7	0.98	3.26	2.86	3.70	4.67	1.21	1.93	1.00	3.26	0.83	3.53
0:100	0	4.88	2.82	3.97	5.48	0.33	2.11	0	4.93		

\* The equations were modified by substituting the ratios which were obtained in this experiment.

If the value for  $g$  is substituted in Equation 1 and it is solved for  $p$ ,

- (6) Pyruvic acid =  $1.1B - 0.1A_1$

By combining Equations 2 and 3, solving for  $g$ , and then multiplying by  $146/88$ ,

- (7)  $\alpha$ -Ketoglutaric acid =  $3.85 (A_2 - A_1)$

From Equations 2 and 3,

- (8) Pyruvic acid =  $3.2A_1 - 2.2A_2$

Similarly, by combining Equations 2 and 4 and solving for  $g$  and  $p$ ,

- (9)  $\alpha$ -Ketoglutaric acid =  $1.9 (A_3 - A_1)$

- (10) Pyruvic acid =  $2.1A_1 - 1.1A_3$

*Method 2*—This method requires only one determination. If the determination is carried out according to Procedure A, then the  $\alpha$ -ketoglutaric acid fraction of  $A_1$  is

$$A_1 \left( \frac{X_{L_{420}:L_{540}} - p_{L_{420}:L_{540}}}{g_{L_{420}:L_{540}} - p_{L_{420}:L_{540}}} \right)$$

$A_1$ , as before, is the total apparent keto acid content, expressed as mg. per cent of pyruvic acid.  $X$ ,  $p$ , and  $g$ , with the subscript  $L_{420}:L_{540}$  are the ratios of the optical densities yielded respectively by the unknown, by pyruvic acid, and by  $\alpha$ -ketoglutaric acid. In all experiments except those shown in Table XIII, the average values were  $p_{L_{420}:L_{540}}$  1.46,  $g_{L_{420}:L_{540}}$  2.06. Then

$$(11) \quad \text{Pyruvic acid} = A_1 \left( 1 - \frac{X_{L_{420}:L_{540}} - 1.46}{0.60} \right)$$

According to Equation 2, only 95 per cent of  $\alpha$ -ketoglutaric acid, expressed as mg. per cent of pyruvic acid, is determined. Therefore,

$$(12) \quad \alpha\text{-Ketoglutaric acid} = 2.9A_1 (X_{L_{420}:L_{540}} - 1.46)$$

If the determination is carried out by Procedure B, the  $\alpha$ -ketoglutaric acid fraction of  $B$  is

$$B \left( \frac{X_{L_{420}:L_{520}} - p_{L_{420}:L_{520}}}{g_{L_{420}:L_{520}} - p_{L_{420}:L_{520}}} \right)$$

The average ratios obtained with 3 cc. of xylene solvent were  $p_{L_{420}:L_{520}}$  1.33,  $g_{L_{420}:L_{520}}$  1.74. Therefore,

$$(13) \quad \text{Pyruvic acid} = B \left( 1 - \frac{X_{L_{420}:L_{520}} - 1.33}{0.41} \right)$$

#### *Direct Method; Total Hydrazones*

This procedure is more specific than may be inferred from a perusal of the effect of the substances listed above under this method. Fortunately, blood and urine contain few of these compounds. The effect of slowly reacting substances, which probably are dicarboxylic keto acids, is minimized by reducing the period of incubation to 5 minutes. The use of Filter 520, as we have shown, also increases the specificity (see Table XII and Fig. 2). When applied to blood and urine (Table VI), this procedure always gave higher results than the extraction method. However, in the case of blood, the difference was relatively small and quite constant. Thus, in the analysis of 67 samples of blood from forty normal subjects the difference between the direct and the extraction methods, expressed as mg. per cent of pyruvic acid, varied from 0.12 to 0.69, with an average difference

of 0.41. The pyruvic acid content, as determined by the extraction method, varied from 0.44 to 5.50 mg. per cent. Since the difference is small com-

TABLE XIV

*Color-Producing Hydrazones of Blood After Ingestion of Dextrose*

The results are expressed in mg. per cent.

After ingestion of sugar	Mr. H.				Mrs. R.			
	Sugar	Total hy- drazones	Pyruvic acid	Other hy- drazones (by differ- ence)	Sugar	Total hy- drazones	Pyruvic acid	Other hy- drazones (by differ- ence)
<i>hrs.</i>								
Fasting	143	1.50	0.90	0.60	90	1.39	0.87	0.52
0.5	224	1.54	0.98	0.56	123	1.37	0.94	0.43
1	302	2.19	1.57	0.62	150	1.67	1.30	0.37
2	280	2.42	1.78	0.64	123	1.89	1.40	0.49
3	223	1.99	1.33	0.66	74	1.76	1.29	0.47
4					80	1.48	1.14	0.34
5					92	1.50	1.10	0.40

TABLE XV

*Color-Producing Hydrazones of Blood after Severe Muscular Exertion*

The results are expressed in mg. per cent.

Subject	Exercise	Lactic acid	Pyruvic acid*	Total hydrazones	Other hy- drazones (by differ- ence)	Lactic acid Pyruvic acid
A	Resting		0.79	1.24	0.45	
"	"		0.69	1.35	0.66	
"	7 to 8 min. after	186	4.72	5.16	0.44	39.4
"	7 " 8 " "	198	4.63	5.04	0.41	42.8
B	Resting		0.91	1.38	0.47	
"	7 to 8 min. after	162	4.25	4.62	0.37	38.1
"	7 " 8 " "	154	4.21	4.38	0.17	36.6
C	Resting		0.69	1.16	0.47	
"	"		0.68	0.93	0.25	
"	7 to 8 min. after	138	5.14	5.79	0.65	26.8
"	7 " 8 " "	125	4.63	5.04	0.41	27.0
D	Resting		0.74	1.28	0.54	
"	"		0.70	1.05	0.35	
"	7 to 8 min. after	85	3.55	4.14	0.59	24.0
"	7 " 8 " "	74	2.96	3.64	0.68	25.0

\* Ethyl acetate solvent, Filter 520.

pared with the pyruvic acid content, the direct method can be used in the clinical laboratory as a rapid, approximate test.

The constancy of this difference under severe conditions is illustrated by

the results given in Tables XIV and XV. It has been shown that the pyruvic acid content of the blood is greatly increased following the ingestion of sugar (28). It is increased to an even greater extent by severe muscular exertion (29-31). The conditions under which the pyruvic acid was produced and the effect on the subject differed greatly in our experiments. Despite these unusual conditions, the differences between the results obtained by means of the direct and extraction methods were no greater than those obtained from normal resting subjects. Furthermore, on the assumption that the changes in the blood represent, qualitatively at least, the changes occurring in the tissues, it may be concluded that demonstrable quantities of aldehydes, methylglyoxal, and free trioses (which readily affect the determination) are not produced under conditions leading to the production of large quantities of pyruvic acid in the normal intact subject.

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# PROTEIN SULFHYDRYL GROUPS AND THE REVERSIBLE INACTIVATION OF THE ENZYME UREASE\*

## THE REDUCING GROUPS OF EGG ALBUMIN AND OF UREASE

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In an addendum to a review article dealing with the rôle of reversible chemical processes in the control of activity of certain enzymes (1), we presented preliminary evidence pointing to some interesting variations in the reactivity of the reducing groups of urease. A closer study promised to reveal information concerning the relationship between the substituent sulfhydryl groups of certain enzymes and the mechanism of their activation and inactivation. The problem appeared also to bear upon apparent anomalies in the chemistry of certain proteins that have been assumed to contain substituent thiol groupings.

The striking differences in the physical and chemical properties of hen's egg albumin (average molecular weight, approximately 45,000) and crystallized urease (particle weight, 483,000) include certain peculiarities in the behavior of these two proteins toward sulfhydryl-detecting reagents. Solutions of native, recrystallized egg albumin do not respond to certain tests for sulfhydryl groups and are notably inert toward porphyrindin; after denaturation of the albumin with guanidine hydrochloride (2), the thiol groups are readily titratable with porphyrindin or ferricyanide (3). Urease, on the other hand, appears to contain substituent sulfhydryl groups which continue to respond to the nitroprusside test after repeated recrystallization of this protein-enzyme (4). The reactive sulfhydryl groups so detectable were reported by us (1) to be titratable with porphyrindin *without loss in the enzymatic activity*; however, a treatment of the urease with guanidine hydrochloride was accompanied by enzymatic inactivation and the appearance of several times the quantity of chemically reactive reducing groups originally titratable. The elucidation of these effects has been assisted by quantitative studies now to be described.

*Urease with Standard p-Chloromercuribenzoate*—Mercaptide-forming re-

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† Henry Strong Denison Scholar for 1940-41.



agents, such as phenylmercuric hydroxide and its derivatives, have been of exceptional service in this laboratory (5) in the study of certain of the sulfhydryl enzymes,<sup>1</sup> namely urease, succinic dehydrogenase,<sup>2</sup> an enzyme of papaya (6), and one or more catalysts in the glycolytic mechanism (7), including possibly triose phosphate dehydrogenase (8). For quantitative studies, *p*-chloromereuribenzoic acid,  $\text{Cl}-\text{Hg}-\text{C}_6\text{H}_4-\text{COOH}$ , is available as a standard reagent. When solutions of urease, sufficiently concentrated and purified, are treated with this reagent (*e.g.*, as the 0.001 *M* sodium salt) and the effect upon the ureolytic activity ascertained, there are disclosed significant facts bearing upon the protein thiol groups in relation to reversible enzymatic inactivation. The procedure is detailed in the experimental part; it amounts to a series of discontinuous titrations of the buffered enzyme, with activity as the "end-point." Under the test conditions, the activity is directly proportional to the concentration of the enzyme.

For facility of comparison, the measures of unitage employed by Sumner (9-11) are used. 1 gm. of urease is taken as equivalent to 130,000 units. Urease has been recrystallized to an activity not exceeding approximately 133,000 units per gm. (10). Elementary analyses have been made upon this material, which, however, is not necessarily a completely "pure" protein (11). Urease possessing an activity of 100,000 units per gm. may be crystallized (9), and such material, or enzyme of somewhat lower purity, was found suitable for all of the purposes of this investigation except, perhaps, the determination of the total sulfhydryl content of the guanidine hydrochloride-denatured enzyme. It was found inadvisable here to attempt the laborious and somewhat uncertain (11) preparation of large quantities of purer material with the source material at present available.<sup>3</sup>

The inactivation curves based upon the use of 0.001 *M* *p*-chloromereuribenzoate are illustrated by Fig. 1. The data refer to Preparations A, B, and C of Table I. In general confirmation of the earlier results (1), it is seen that a definite amount of the mercury reagent must be added before inactivation begins. The inactivation is complete only after a second stoichiometrically comparable portion of *p*-chloromereuribenzoate has been used. From a number of determinations of this kind with independent, potent preparations of urease (Table I), it appears that 2 moles of *p*-

<sup>1</sup> The term refers to those protein-enzymes the activity of which may be modified, often reversibly, through certain chemical actions upon their substituent sulfhydryl groups. Their characterization from this point of view necessarily involves a variety of collateral evidence (1, 5); however, for reasons which appear in this paper and in the references cited, we consider the application of organometallic compounds of the type,  $\text{R}-\text{Hg}-\text{X}$ , to have been especially and almost uniquely valuable in such a characterization.

<sup>2</sup> Pohl, H. A., and Hellerman, L., unpublished work.

<sup>3</sup> We are grateful to Dr. David B. Sabine, The Arlington Chemical Company, for helpful cooperation in the procurement of suitable jack bean meal.

chloromercuribenzoate would be required for the inactivation of some 21,000 to 22,000 gm. of urease, on the basis of the equivalence of 1 gm. to 130,000 units. From these observations it might be considered that, for this amount of the enzyme, the first residue weight of protein sulfhydryl to combine is not to be correlated *directly* with the enzymatic function, only the second apparently being so concerned. Observations with other reagents later to be described permit an extension of this interpretation. For convenience, the two categories of sulfhydryl groups may be designated respectively as the *a* and *b* groups. The addition of the second equiv-

TABLE I  
Urease Preparations, Gm. of Urease Equivalent to 2 Moles of *p*-Chloromercuribenzoate ("Hg")

Preparation	Units per gm. protein	Units per ml.	"Hg," 0.001 M, = <i>a</i> + <i>b</i> groups*	Urease† = 2 moles "Hg"
			ml.	gm.
A	99,200	2670	1.92	21,300
			1.80	22,800
B	105,000	1900	1.40	20,900
			1.30	22,400
C	89,200	2810	2.10	20,600
D	86,900	2110	1.70	18,400
E	84,000	2740	2.00	21,000
F	82,000	2810	2.00	21,500
G	75,300	2470	2.20	17,200
H	70,700	2050	2.00	15,800
I	65,000	2640	2.00	20,000
J	55,000	1950	1.90	15,800
K	32,000	710	1.02	10,800

\* The quantity of "Hg" solution required for the inactivation of the urease in 1 ml. of preparation of the unitage and purity given respectively in the third and second columns; see the text and the experimental section.

$$\dagger = \frac{2 \times 10^5 \times (\text{units per ml.})}{(\text{ml. } 0.001 \text{ M "Hg"}) \times (130,000)}$$

alent of *p*-chloromercuribenzoate is accompanied by certain physical changes in the reaction mixtures that will require study by ultracentrifugal and other methods. It is to be emphasized that the phenomena here described are observed in most clear cut fashion with concentrated solutions of sufficiently purified enzyme as shown in Table I; with such preparations (Preparations A to F), the "equivalent weight" approaches

\* It will be interesting to ascertain the equivalent weight with urease of maximum purity. However, the trend in Table I suggests that in concentrated solutions of highly purified urease (e.g., Preparations A and B) the small amount of extraneous protein (denatured, oxidized urease?) has not interfered seriously in the quantitative processes used.

21,000 to 22,000. However, the data with even relatively crude material are found to reflect the two-step character of the inactivation, as well as the apparent stoichiometric equivalence of the *a* and *b* categories. Somewhat low equivalent weights are obtained, but, nevertheless, the back titration curves with standard cysteine<sup>5</sup> solutions are found to depict the usual reversal of inactivation (Preparation K, Table I; Fig. 2).

*Urease Reducing Groups*—The relationship of the *a* and *b* sulfhydryl groups is confirmed by various experiments, presently to be described,

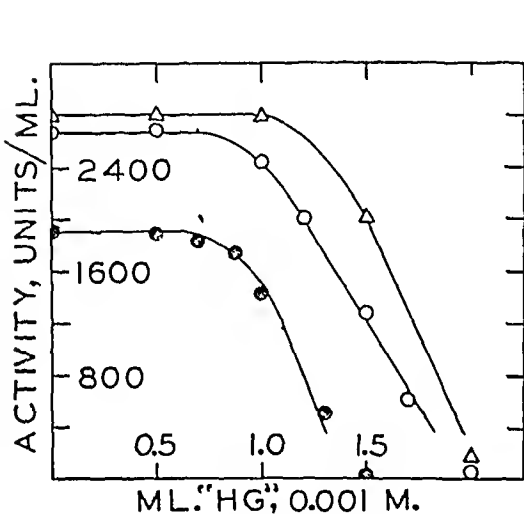


FIG. 1

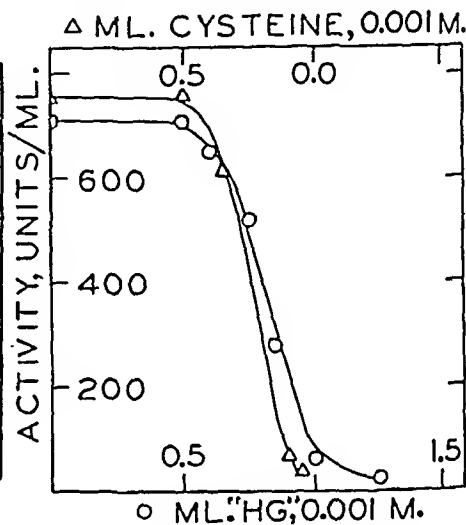


FIG. 2

FIG. 1. Inactivation curves. See the text, especially "Inactivation of urease with standard *p*-chloromercuribenzoate" under the experimental section. O, ●, and Δ refer to data for Preparations A, B, and C respectively (Table I). "Hg" denotes sodium *p*-chloromercuribenzoate solution.

FIG. 2. Inactivation and reactivation of an impure preparation. Preparation K, Table I; see the text, especially "Reactivation with cysteine." O denotes inactivation; Δ, back titration with cysteine.

with iodoacetamide, porphyrindin, and iodoso compounds. If elementary analyses (10) upon highly purified urease are taken to represent the figures for the pure enzyme, then it is calculated readily that a total of 8 gm. atoms

<sup>5</sup> These data afford presumptive evidence that the cysteine-(S)-mercury compound is even less dissociated than the corresponding protein mercaptide. It is a matter of great interest that cysteine not only restores, but, in addition, frequently enhances the activity of highly purified preparations (about 10 per cent). The full discussion of this phenomenon must await consideration of the rôle of the substituent sulfhydryl groups of certain enzymes in the actual catalytic processes concerned.

of sulfur would be present in 21,300 gm., a figure close to 21,000 to 22,000. If, in addition, it may be assumed that our results from *o*-iodosobenzoate titrations<sup>6</sup> of urease denatured by guanidine hydrochloride reflect the total sulfhydryl content of the urease molecule, we would have the following relationship. Of the 8 gm. atoms, 5 would represent the total sulfhydryl sulfur in which are included the 2 residue weights comprising the *a* and *b* categories. The number 5 for total sulfhydryl in denatured urease is of somewhat uncertain significance, but the recurrence of this value in the *o*-iodosobenzoate titration of different samples of somewhat impure urease, and the finding by porphyrindin estimations of 4 to 5 reducing equivalents in such samples suggested that we have at least an upper limit for the total sulfhydryl content, which appears thus to exceed by a considerable margin the 2 atoms of sulfhydryl sulfur detected in the combining unit<sup>7</sup> by *p*-chloromercuribenzoate.

*Quantitative Studies with Other Reagents*—Urease of the potency and purity used in these studies<sup>8</sup> is not inactivated after being treated with 3 to 10 equivalents of dilute porphyrindin per minimum combining unit (21,300 gm.). However, the dye evidently has reacted with the *a* groupings, for the enzyme is inactivated quantitatively by the subsequent addition of 1 molecular equivalent of *p*-chloromercuribenzoate, instead of the 2 required when the mercury reagent is used alone. The activity is recovered as usual when cysteine is added subsequently. Similar results are obtained when buffered urease is treated with an equivalent of *o*-iodosobenzoate. However, if the urease is treated with an excess of concentrated iodoso reagent, the latter appears to attack the *b* groupings also: an inactivation that is only partially reversible occurs. Comparable results are obtained with iodoacetamide, the interaction of which under suitable conditions with protein sulfhydryl groups appears to be one of alkylation in the sense,  $R-SH + I-CH_2-CO(NH_2) \rightarrow R-S-CH_2-CO(NH_2) + H^+ + I^-$ . Sufficiently dilute iodoacetamide may attack only the *a* groups, as indicated again by estimation of the amount of *p*-chloromercuribenzoate required for reversible inactivation after the action of iodoacetamide has been permitted to take place. With more concentrated iodoacetamide (0.05 *M*), the urease is inactivated directly and

<sup>6</sup> See "Experimental."

<sup>7</sup> On the basis of diffusion rate experiments (12) Hand finds that units of urease with particle weight as low as 17,000 may possess ureolytic activity. This result cannot now be interpreted in relation to our work; the minimum combining weight evaluated in our experiments is close to 21,000 gm.

<sup>8</sup> Certain samples of impure urease were found (1) to be inactivated *partially* by relatively large amounts of porphyrindin. However, it appears from the present work that urease of an activity of 100,000 units (9-11) per gm. is rather resistant to inactivation when treated with even a large excess of this oxidizing agent.

irreversibly, as described in the experimental part and in accordance with the observations of Smythe (13). These results strikingly illustrate the utility of *p*-chloromercuribenzoate as a diagnostic reagent in connection with the urease sulfhydryl groups, and they afford interesting support for the differentiation of the *a* and *b* categories.

Additional observations with various reagents are detailed in the experimental part.

#### DISCUSSION

This study has presented direct evidence in general support of the correlation of the control of activity of certain enzymes through reversible chemical processes and the rôle of specific protein sulfhydryl groupings. The approach has been more quantitative than has hitherto been possible. The work has involved a critical examination of the effect of various reagents upon urease, which, thanks to the efforts of Sumner and his students, is the sulfhydryl protein-enzyme at present most readily available in a purified state. A careful choice had to be made of reagents suited to the study; these have included (1) organic mercaptide-forming salts, (2) oxidizing agents, in particular porphyrin oxides, a porphyrindin, and *o*- and *p*-iodosobenzoates, and (3) an alkylating reagent, iodoacetamide. The results bear upon the factors controlling the reactivity of certain protein groupings.

For example, the apparent differences in chemical reactivity among the urease sulfhydryl groups suggest functional differences. It has seemed not implausible that the *a* category of groupings most readily attacked by dilute solutions of porphyrindin, *o*-iodosobenzoate, and iodoacetamide may be substituted close to the "surface" of the protein molecule, being thus readily available to the action of these reagents; the *b* category, which would seem to be concerned more directly with control of enzyme activity (and possibly also with the mechanism of the catalysis), is protected to an important degree from the action even of powerful oxidizing agents. Reversible enzymatic inactivation is secured only after 2 residue weights of —SH for approximately 21,300 gm. of protein have interacted with *p*-chloromercuribenzoate, under proper conditions, or with certain oxidizing or alkylating reagents followed by 1 equivalent of the mercuribenzoate. Beyond the first 2 —SH residue weights are the additional potentially reducing groups, revealed by assays for total sulfhydryl in denatured urease. The observation of these relations depends upon a careful control of experimental conditions, as detailed in the experimental part. Such differences in thiol reactivity are remindful of the variations in the properties of the pepsin protein amino groups, so clearly demonstrated by Northrop and his collaborators (14).

A certain unpredictability in the behavior of sulfhydryl enzymes toward various reagents has introduced confusion in several aspects of the subject. For example, the *seeming* inertness (on the basis of activity measurements as sole criterion) of highly purified urease toward diluted porphyrindin, iodoacetamide, and ferricyanide ion is highly contradictory to other evidence if consideration of the quantitative and structural matters discussed in this paper is neglected. Such difficulties as those considered by Bergmann and his collaborators (15) in connection with the applicability of the sulfhydryl theory to the activation of enzymatic constituents of crude papain which promote the hydrolysis of certain synthetic substrates (*e.g.*, benzoyl-*L*-argininamide) may not be particularly apposite to the present discussion. It may be recalled that the papain constituent that has been crystallized (16) by Balls and Lineweaver and named by them crystalline papain has been found to function as catalyst in the hydrolysis of hemoglobin apparently quite without benefit of any external activator or co-enzyme, and indeed to remain partially active in the presence of porphyrindin, a reagent known to oxidize cysteine and HS—glutathione. That crystallized papain is rather resistant to the action of porphyrindin (*which conceivably might be held immobilized upon the protein surface*) and nevertheless appears to be inactivated by 1 molecular equivalent of iodoacetate ion (17) may reflect merely the relative structural availability (1) of that sulfhydryl grouping, which in papain has been correlated with inactivation, toward these two reagents *under the conditions used*.

In crystallized albumin of hen's eggs, the sulfhydryl groups appear to be even less reactive or available. However, the results from Anson's extensive experimentation (3) give warning that we may be dealing here merely with marked differences in degree. Anson has shown that certain reagents (*e.g.*, iodine with potassium iodide at pH 7) can oxidize sulfhydryl groups of native egg albumin seemingly without denaturation. It may be pointed out, in this connection, that with iodine (plus iodide ion), or with oxygen in the presence of a trace of cupric ion, it has been found possible to secure inactivations of urease and of papain (5) that have been more or less reversible. Moreover, in the present investigation, with *o*-iodosobenzoate there again have been observed inactivations of urease that usually were partially reversible and occasionally were extensively so. There is little question, however, that significant differences exist in the behavior of the thiol groups of urease and of egg albumin. For present purposes it may be concluded that these egg albumins contain no reactive, surface-substituted thiol groups comparable with the urease groups. However, the destruction of the integrity of the surface of either protein, by the action of concentrated guanidine hydrochloride or by other appropriate means, makes readily available to the action of most

reagents that interact with cysteine sulfhydryl those reducing groups which, in the intact molecule, are "protected" to a greater or less degree.

### EXPERIMENTAL

#### *Quantitative Studies of Purified Urease with p-Chloromercuribenzoate*

*Preparation of Urease*—The methods of crystallization used were those of Sumner and his collaborators (9-11), or modifications of these. Large quantities of the enzyme were required, for the isolation of which the quality of jack bean meal available on the market was not always suitable. The purification of sufficient urease constituted a troublesome problem, involving often repeated recrystallizations, with routine assays of ureolytic activity and micro-Kjeldahl determinations of protein and non-protein nitrogen upon the fractions. In one of the more favorable preparations, 800 gm. of jack bean meal, the activity of which had been found to be 200,000 units per kilo, were divided into 200 gm. portions, and each portion in succession was extracted at room temperature (22°) for 10 minutes, with the aid of constant, gentle stirring, in 1 liter of 31.5 per cent acetone (analytical reagent; found by test to be aldehyde-free). The mixture was filtered with suction on a single thickness of filter paper (Whatman, No. 41) in a Buchner funnel until the residue was practically dry, no air being drawn through during the process. The volume of filtrate from each 200 gm. portion (approximately 800 ml.) was made up to 950 ml. by the addition of pure acetone. The resulting cloudy solution was allowed to stand overnight at 0°, after which it was centrifuged in the cold room. The supernatant liquid had an activity of less than 1 unit per ml. and was discarded. The precipitates were pooled, and treated with 10 ml. of redistilled water. After being stirred, this mixture was centrifuged, leaving 7 ml. of supernatant having an activity of 200 units per ml. The residue was treated with 20 ml. of water, allowed to stand for some hours, during which the mixture was stirred occasionally, and then centrifuged. There were obtained 39 ml. of supernatant liquid of ureolytic activity, 1000 units per ml. For crystallization, there were added to the supernatant 5 ml. of phosphate buffer (Buffer A, 53.05 ml. of  $\text{M NaOH}$  and 125.0 ml. of  $\text{M KH}_2\text{PO}_4$ , made to 250 ml. with redistilled water; at pH 7 when diluted 1:10), in order to have buffered enzyme solution of pH 7, and then 22 ml. of pure acetone. After this mixture stood overnight at 0°, it was centrifuged and the solid extracted with water, giving 21.5 ml. of urease solution, activity 1460 units per ml. A further reprecipitation and re-solution of the solid in water yielded 5 ml. of solution having activity of 2470 units per ml. and per gm. of protein 75,300 units, with negligible non-protein nitrogen. Any urease-rich residues from the above operations were treated further in similar fashion. A final recrystallization of material of purity

of 72,000 to 75,000 units per gm. of protein was effected by the addition to the concentrated, chilled urease solution of enough pure acetone to make the solution 31 per cent with respect to acetone, followed by the dropwise addition of chilled, concentrated phosphate buffer (pH 7) until there was a faint, permanent turbidity, after which the solution remained at 0° for 15 hours. The crystals that had formed were collected at the centrifuge and dissolved in water to give a product having an activity of 1900 units per ml. and 105,000 units per gm. of protein.

In this run, there were recovered from 160,000 units in the jack bean taken 94,000 units, accounted for as highly purified urease, as concentrated urease solutions of lesser purity, and as dilute solutions. Some of the runs were less successful, and some commercial offerings of jack bean flour were such that numerous laborious reprecipitations and recrystallizations were required for the production of very small amounts of good urease. The pH number of our filtrates from the initial jack bean extractions was close to 7; in recrystallizations, we buffered the solutions to pH 7, at which there appeared to be less denaturation and inactivation than in more acid regions. Purified urease solutions remained at 1-5° for several weeks without diminution in the activity.

*Estimation of Activity*—Ureolytic activity was determined in reaction mixtures of pH 7 at 20.0°, as described by Sumner (9-11). The ammonia formed by hydrolysis of urea was estimated by means of aeration for 30 minutes into 0.02 N hydrochloric acid, the excess acid being titrated in CO<sub>2</sub>-free air with chlor-phenol red as indicator.

*Standard Sodium p-Chloromercuribenzoate Solution*—*p*-Chloromercuribenzoic acid, Cl—Hg—C<sub>6</sub>H<sub>4</sub>—COOH, was prepared by means of the oxidation of *p*-tolylmercuric chloride with alkaline potassium permanganate (18). The well washed product was dried and analyzed (calculated for C<sub>7</sub>H<sub>5</sub>O<sub>2</sub>ClHg, Hg 56.2; found, Hg 56.0, 56.4 per cent). For the preparation of standard solutions of the sodium salt, an exactly weighed portion of the powdered acid was dissolved in a slight excess of N NaOH followed by dilution to volume. The calculated molarity of solutions so prepared agreed satisfactorily with the results of iodometric determinations based upon the reaction represented by Cl—Hg—C<sub>6</sub>H<sub>4</sub>—COO<sup>-</sup> + I<sub>2</sub> + 3I<sup>-</sup> → I—C<sub>6</sub>H<sub>4</sub>—COO<sup>-</sup> + HgI<sub>4</sub><sup>-</sup> + Cl<sup>-</sup>.

*Inactivation of Urease with Standard p-Chloromercuribenzoate*—Urease solution (0.100 or 0.200 ml.) was introduced into a dry flask. To this was added phosphate buffer of pH 7 (Buffer A, 1:10, preparation of urease), usually 2.00 ml., followed by an exactly measured quantity of 0.001 M *p*-chloromercuribenzoate. The homogeneous mixture was allowed to stand at 20.0° for at least 10 minutes, after which the determination of ureolytic activity was carried out with the use of the usual urea-phosphate reaction mixture.



In the case of each independent preparation of purified urease, a sufficient number of such determinations, with appropriate variation in the amount of *p*-chloromereuribenzoate added, was accomplished to secure a quantitative delineation of the inactivation relationship (*cf.* Fig. 1). The results are calculated to and expressed in terms of units of urease activity found per ml. of urease taken. Such data are given in Table II for Prepa-

TABLE II

*Inactivation of Urease with Standard Sodium p-Chloromereuribenzoate\**

Preparation A, crystalline urease, 99,200 units per gm. of protein (micro-Kjeldahl; N in urease taken as 16.0 per cent (10).) The units per ml.† were 2670. In each determination 0.100 ml. of urease and 2.00 ml. of Buffer A (1:10) were used; see the text.

	0.001 M <i>p</i> -chloromereuribenzoate added to 0.100 ml. urease in each determination							
	0.000 ml.	0.050 ml.	0.100 ml.	0.120 ml.	0.150 ml.	0.170 ml.	0.200 ml.	0.300 ml.
	units	units	units	units	units	units	units	units
Activity per ml. urease . . .	2670	2690	2440	2010	1280	610	60	20

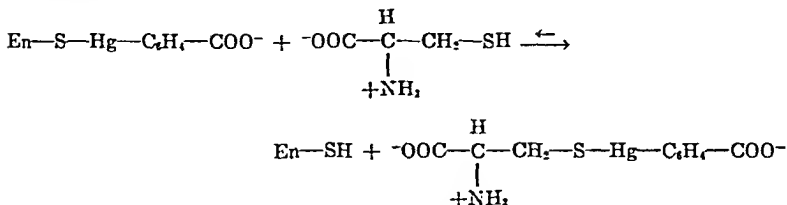
\* As illustrated in Fig. 1, the results may be plotted with units per ml. found as ordinate and ml. of 0.001 M *p*-chloromereuribenzoate per ml. of urease as abscissa. From such a plot of the above data, the "equivalent weight" of urease may be evaluated as follows: Urease, 1 gm. taken  $\approx$  130,000 units. Then if in 2670 units of urease,  $a \approx b$  groups  $\approx$  0.96 ml. of 0.001 M *p*-chloromereuribenzoate, gm. of urease  $\approx$  1 mole of the mercuribenzoate for each category would be 21,300; if  $a \approx b$  groups  $\approx$  0.90 ml., gm. of urease  $\approx$  1 mole would be 22,800; see also the calculation in Table I. Redeterminations of the "titration curve" gave consistent results. A third redetermination was conducted upon Preparation A 1 week later than the first two. The initial activities found were, respectively, 2670, 2690, 2690.

† With solutions of crystalline or highly purified urease the stoichiometric relations in the *p*-chloromereuribenzoate determination are not substantially altered when the urease concentration is changed. Thus, when 0.050 ml. of urease was substituted for 0.100 ml. in this series of tests, the activity found *per ml.* of urease was 2680 units, and there was determined  $a \approx b$  groups  $\approx$  1.00 ml. of 0.001 M mercuribenzoate reagent.

ration A. The results with eleven different preparations of urease of varying purity and potency are summarized in Table I.

*Reactivation with Cysteine*—Cysteine hydrochloride, after being dried in a vacuum desiccator containing beakers of phosphorus pentoxide and flaked sodium hydroxide, usually is found to give slightly high values for sulphydryl sulfur. For reactivation experiments, the dried salt may be made to approximately 0.002 M in air-freed water, and then analyzed by the iodoso method (19). Any addition of this solution to *p*-chloromereuribenzoate-treated urease is accompanied by the addition of an equivalent amount of sodium carbonate.

The addition to buffered, mercuribenzoate-inactivated urease solution of cysteine, equivalent to or in excess of the amount of mercuribenzoate added after the addition of the initial, non-inactivating portion ( $\approx a$ ) of the latter reagent, resulted in the restoration of the ureolytic activity. If the enzyme-mercury combination (e.g., for  $b$  groups) is designated as  $\text{En-S-Hg-C}_6\text{H}_4\text{-COO}^-$ , the over-all process may be represented by the equation



Any excess mercuribenzoate reagent in the reaction mixture would also combine with cysteine to yield the last designated compound (mercaptide salt). Reactivation by the action of cysteine upon urease inactivated with  $p$ -chloromercuribenzoate was secured without exception, complete restoration of activity being observed. Frequently the activity was slightly greater than the initial. Even impure urease of sufficient potency could be titrated back, as shown in Fig. 2. For this determination, a series of buffered mixtures containing 0.100 ml. of Preparation K and 0.100 ml. of 0.001  $M$  mercuribenzoate was treated with 0.001  $M$  cysteine hydrochloride in amounts varying from 0.005 to 0.100 ml. Apparently the protein impurities in such material as Preparation K cause distortion in the stoichiometry involved in the urease-mercury relationship.

*Effect of Sodium  $o$ -Hydroxymercuribenzoate*— $o$ -Hydroxymercuribenzoic "anhydride" was prepared (20) by mercuriation of benzoic acid with mercuric acetate. A solution of the sodium salt was desired in order that the action of the ortho-substituted compound upon urease might be compared with that of  $p$ -chloromercuribenzoate. A titration curve was constructed upon Preparation E, with the use of the  $o$ -mercuribenzoate, and was found to be substantially the same as that given with  $p$ -chloromercuribenzoate (Table I). Cysteine reactivation also was demonstrated. The two mercury compounds display no observable differences in their interaction with the  $a$  and  $b$  sulphydryl groups of the urease molecule.

#### *Quantitative Studies with Porphyrindin, Iodosobenzoates, Iodoacetamide, and Certain Other Reagents*

*Porphyridin*—Because the synthesis of porphyridin (21, 22) is somewhat troublesome (23) and its solutions are decidedly unstable, this reagent is often inferior in usefulness to iodine, ferricyanide, and especially  $o$ -iodoso-

benzoate (19). In our view, its almost unique value lies in its selective action with respect to certain categories of protein sulfhydryl groups. There is no observable action with recrystallized, *native* hen's egg albumin, and any action with the sulfhydryl of crystalline papain would appear to be slow and incomplete (16, 17). On the other hand, in the case of purified urease, a portion of the sulfhydryl content (the *a* groups) is highly sensitive, being substituted apparently near or at the protein surface; this fraction is oxidized rapidly by porphyrindin, which, however, even in excess does not extensively attack the *b* groups with accompanying enzymatic inactivation.

*Treatment with Porphyrindin; Subsequent Titration with p-Chloromercuribenzoate*—Porphyrindin may be kept for a long period as the solid substance at 0–10° in a desiccator charged with calcium chloride. For the

TABLE III  
*Purified Urease with Porphyrindin*

Preparation A, urease, 0.100 ml.; Buffer A, pH 7. Porphyrindin, 0.001 N; *p*-chloromercuribenzoate, 0.001 M.

Porphyrindin, ml.....	0.00	0.088	0.176	0.528*	0.075		0.075	0.075	0.075
<i>p</i> -Chloromercuribenzoate after porphyrindin, ml. . . .						0.090	0.050	0.070	0.100
	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>
Activity per ml. urease.....	2670	2670	2430	2330	2670	2640	2250	1330	890†

\* The blue color of the porphyrindin persisted even after the termination of the digestion period in urea-phosphate.

† Control, cysteine (0.001 M) 0.2 ml. after porphyrindin and *p*-chloromercuribenzoate, 2580.

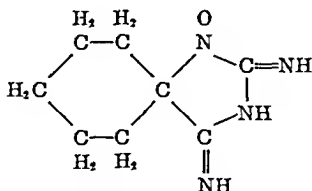
following experiments, a solution was freshly prepared by dissolving 0.020 gm. in 100 ml. of ice water. The blue solution was standardized immediately before and after each experiment by titration against 0.001 N ascorbic acid which had been assayed iodometrically. The ascorbic acid was titrated into iodine formed by the addition of standard iodate to excess sodium iodide and hydrochloric acid.

To urease-buffer mixtures in a graded series were added varying amounts of porphyrindin. As shown by the data of Table III, the enzyme was not inactivated extensively even by a large excess of this oxidizing agent. The addition of *p*-chloromercuribenzoate to urease to which had been added porphyrindin equivalent to three-fourths of the *a* fraction is seen to have produced close to the calculated effect. With cysteine there was obtained the usual reactivation.

With various other preparations of highly purified urease, it was ob-

served<sup>9</sup> that marked inactivation was not obtained when there was added porphyrindin equivalent to the sum of the *a* and *b* groups, or in excess thereof, and the subsequent addition of *p*-chloromercuribenzoate in an amount equivalent only to *a* ( $\approx b$ ) did produce inactivation, reversible by the action of cysteine.

*Effect of an Analogue of Porphyrexide*—This analogue possessing the structure



had been synthesized in the course of work, as yet unpublished, by C. C. Porter and one of us (L. H.) in an effort both to test the generality of the Piloty synthesis and to make available compounds useful in certain studies of proteins. The substance is more stable than porphyrexide; its solutions are evaluated iodometrically. When buffered urease (Preparation A) was treated with this powerful oxidizing agent ( $E'_0$  at pH 7 = 0.690) in 1 to 5 equivalents with respect to the *a* fraction, the full initial enzymatic activity was retained. The subsequent addition of 1 equivalent of *p*-chloromercuribenzoate resulted only in partial inactivation, but we have independent evidence that the porphyreoxides may combine with certain heavy metal compounds.

*Effect of Sodium o-Iodosobenzoate*—The utility of this reagent in the estimation of substances of the cysteine type has been demonstrated (19). Its applicability in assays of certain protein sulfhydryl groupings will be treated presently, and it will be shown that those reactive protein groups that are detectable readily by means of nitroprusside or porphyrindin may be expected also to react rapidly at pH 7 with iodosobenzoate, undergoing oxidation to the dithio state.

The behavior of *potent, purified* urease in the presence of *dilute o*-iodosobenzoate was decidedly clear cut. Of Preparation A, 0.100 ml. was treated with 2.00 ml. of Buffer A (pH 7) and 0.2 or 0.4 ml. of 0.001 *N* sodium *o*-iodosobenzoate solution. The mixture was allowed to stand at 37° for 2 hours, after which the ureolytic activity was determined and found to be the same as the initial activity; namely, 2690 units per ml. of urease. The addition of only 0.100 ml. of *p*-chloromercuribenzoate solution to iodoso-

\* Dr. Sidney Goren kindly conducted some of these experiments.

treated urease (with 0.2 or 0.4 ml. of iodoso reagent) resulted in the complete abolishment of the enzymatic activity.

It was concluded that under these conditions the effect of iodosobenzoate, even in excess, was to oxidize only the  $\alpha$  sulphydryl groups. A number of urease preparations were tested as described above.

*Effect of p-Iodosobenzoate*—Entirely similar results were obtained by treatment of this urease at pH 7 with standardized solutions prepared from *p*-iodosobenzoic acid (24).

*Inactivation in More Concentrated Solutions*—When *o*-iodosobenzoate was added to portions of Preparation A as well as to several other preparations listed in Table I, the *reaction mixtures being kept more concentrated* (total volume of urease, buffer, and iodosobenzoate of the order of 0.4 to 0.8 ml.), a partial inactivation of the urease was observed. The activity could be restored to some extent by the action of cysteine, but these reactivations were infrequently extensive. The results of rate studies, which need not be presented in detail here, suggested forcibly that the irreversible action, under these conditions, of excess (5 to 10 equivalents) iodoso reagent may be a *slow process* requiring 5 to 12 hours for completion and coinciding with a *denaturation* of the urease protein.

*Effect of Iodoacetamide*—This reagent was prepared in acetone solution by the interaction of chloroacetamide and sodium iodide. It was recrystallized from water until pure (3). Dilute aqueous solutions of iodoacetamide were found to be rather stable. No silver iodide was obtained when a solution was treated with silver nitrate and dilute nitric acid and boiled for  $\frac{1}{2}$  minute. Iodide ion was obtained, however, when the solution was treated briefly with hot sodium hydroxide. Iodoacetamide was considered to be an especially valuable reagent in the present investigation, since it is known to alkylate mercapto compounds rather rapidly. Effects of the iodoacetate derivatives upon certain protein groupings other than  $-SH$  most probably are slower.

Mixtures consisting of urease (Preparation A), 0.100 ml., Buffer A (pH 7), 1.00 ml., and iodoacetamide (0.001 M), 0.400 ml., were kept at 37° for 2 hours; activity, 2700 units per ml. of urease; initial activity, 2700. After the subsequent addition of 0.050 ml. of *p*-chloromercuribenzoate, the activity was 1500 units, and after 0.100 ml. it was 60.

After treatment of 0.100 ml. of Preparation A (buffered at pH 7) with only 0.100 ml. of iodoacetamide (0.001 M) at 37° for 2 hours, the activity was found to be 2600 units per ml. of urease; the subsequent addition to such a mixture of 0.100 ml. of *p*-chloromercuribenzoate (0.001 M) effected a reduction in the activity to 100 units per ml.; treatment of 0.100 ml. of urease with 0.050 ml. of the iodoacetamide in the same way, followed by the addition of 0.150 ml. of the mercury reagent, was found to have brought

the activity to 100 units per ml. of urease. Complete restoration of the activity was accomplished with the use of cysteine. When to 0.100 ml. of urease were added 0.100 ml. of iodoacetamide and *immediately* thereafter 0.100 ml. of the mercury reagent, the activity remained at 2600 units.

*Entirely consistent data* were obtained in similar experiments with Preparations E and I.

It may be concluded, therefore, that *under the conditions* the *a* groups of urease of the purity cited are slowly and completely acted upon in the presence of 1 equivalent of iodoacetamide, and that a 3-fold excess of the latter reagent does not noticeably attack the *b* groups.

When, however, the iodoacetamide in considerable excess is permitted to act in more concentrated solutions (*e.g.*, 0.025 M), the effect is more drastic, enzymatic inactivation taking place increasingly even at room temperature. The following is illustrative and typical. Of Preparation I (2650 units per ml.), 0.100 ml. was treated with 1 ml. of Buffer A and 0.200 ml. of 0.001 M iodoacetamide. The mixture was allowed to stand 2 hours at 37°, after which a test showed that the activity was unchanged; the addition to such treated urease of 0.110 ml. of 0.001 M *p*-chloromercuribenzoate caused a decrease to 100 units per ml. However, in a similar test in which 0.1 ml. of 10 per cent iodoacetamide was used, the activity after 1 hour at 30° was found to be 1280 units per ml. of urease.

Under the latter conditions, the *b* groupings presumably were also attacked.

*Effect of Certain Other Reagents*—Treatment of portions of purified urease, suitably buffered at pH 7, with cystine or with dithiodiglycolic acid,  $(\text{HOOC}-\text{CH}_2-\text{S}-)_2$ , resulted in no loss in activity.

These results appear to be entirely consistent with the concept of differentiation in the activity and availability to the action of certain reagents of the *a* and *b* groupings. The *a* groups *might* have been oxidized to some extent.

The action upon the *a* groups of ferricyanide ion ( $\approx a \approx b$  groups) under the usual conditions was found to be decidedly slow (*p*-chloromercuribenzoate technique). In harmony with previous observations (5) with this ion, no inactivation was observed.

#### *Estimation of Reducing Groups of Denatured Urease and Egg Albumin; Applicability of o-Iodosobenzoate*

*Guanidine Hydrochloride-Denatured Egg Albumin with Oxidizing Reagents.*  
*Introduction*—In recent papers Greenstein (2) and Anson (3) have considered in detail the subject of estimation of the reducing groups of denatured sulfhydryl proteins. Greenstein has extensively treated the method which involves the titration of guanidine hydrochloride-

denatured protein with porphyrindin. Anson has studied carefully methods concerned with the use of various denaturants, including certain useful detergents, and the applicability of ferrieyanides and of iodine. Because porphyrindin is not a specific reagent and possesses some oxidative activity with respect to free and combined tyrosine, Barron *et al.* (25) have cautioned concerning its use in testing for sulfhydryl groups in proteins. For the same reason its use as a titrimetric reagent with proteins has been criticized (26). However, Greenstein and Jenrette (2) have shown how this objection may be met substantially in quantitative work; they have indicated also that there is rather good agreement in the results of estimations of the sulfhydryl groups of denatured egg albumin with a wide variety of reagents. As stated earlier, we have found porphyrindin to be of special utility as an aid in the differentiation of the reactive *a* and the *b* fractions in *active* urease. It has been used also in the estimation of the total reducing groups of denatured urease (see below).

For the purposes of this investigation, we wished to have in hand for the estimation of protein sulfhydryl groups a less unstable oxidizing reagent well adapted to use in a method possessing satisfactory precision. Such a reagent was found in *o*-iodosobenzoic acid. We already (19) have demonstrated the value of this substance in the estimation of cysteine, and have discussed in this paper a specific application with urease. We cite now some data which indicate the character of results obtained with denatured egg albumin.

*Procedure*—Hen's egg albumin was prepared by the method of Kekwick and Cannan (27). It was recrystallized three times, dissolved in water, and dialyzed against redistilled water until sulfate-free. The protein content was estimated by means of micro-Kjeldahl determinations, the ratio of albumin to nitrogen being taken as 6.45. The perfectly clear solutions were negative to nitroprusside and did not decolorize porphyrindin.

The sulfhydryl content of denatured albumin was estimated as follows: Mixtures each consisting of 1.00 ml. of egg albumin (3.59 per cent), 2.0 ml. of *M* phosphate buffer (pH 7), and 2.0 gm. of pure guanidine hydrochloride were allowed to stand under nitrogen at 22–30° for 30 minutes. Each mixture then was treated with 20.00 ml. of sodium *o*-iodosobenzoate (0.001634 *N*) and allowed to stand 2 minutes, after which there was added a freshly prepared solution of 1.0 gm. of sodium or potassium iodide in 5.0 ml. of *N* hydrochloric acid, and the iodine was titrated with 0.001764 *N* sodium thiosulfate. In four independent determinations, the thiosulfate required was 16.32, 16.38, 16.32, and 16.38 ml. The data indicate 1 reducing equivalent per 9000 gm. of protein, or 5 equivalents ( $\approx$  5 residue weights of —SH) per 45,000 gm., a figure that has been found to be close to the average molecular weight of the albumin (28). The reducing

capacity of the denatured albumin may be calculated in terms of cysteine; found, 1.29 per cent "cysteine." Determinations were made upon three different preparations of crystallized egg albumin denatured with guanidine hydrochloride; the reducing capacity of each was found to be close to the result cited.

It has been found advisable (*cf.* (19)) to carry out the estimations with iodoso reagent upon a series of the protein denaturation mixtures, varying amounts of *o*-iodosobenzoate being used in order to determine a minimum workable excess of the oxidizing agent. The volumes are kept the same in all determinations. From the data, an essentially linear curve may be constructed, suitable extrapolation permitting evaluation of the protein —SH content.

Inasmuch as the step in the analytical procedure following the addition of *o*-iodosobenzoate involves the reduction of excess oxidizing reagent with acidified iodide and the iodine formed would itself oxidize protein sulfhydryl, if present, it was necessary to demonstrate, as was done in the case of cysteine (19), that the oxidation of the sulfhydryl groupings actually was accomplished by *o*-iodosobenzoate before the addition of iodide. This was proved conclusively as follows: Samples of albumin, denatured as described above, were found to give vivid nitroprusside tests and also readily to decolorize 0.001 *N* porphyrindin. However, after such preparations were treated at pH 7 with excess *o*-iodosobenzoate solution in the manner described, they were found to be nitroprusside-negative, and to possess no further reducing action with respect to added porphyrindin. Entirely concordant results were obtained in parallel tests with cysteine in place of the denatured protein.<sup>10</sup>

*Iodosobenzoate Method Applied to Reduced Glutathione*—(See also (19)). The following is representative of a number of determinations upon different high grade samples of HS—glutathione. Each of four 10.00 ml. portions of glutathione solution was treated with *M* sodium phosphate of pH 7 and 20.00 ml. of *o*-iodosobenzoate, 0.001643 *N*; after  $\frac{1}{2}$  minute, acidified sodium iodide was added. The ml. of 0.001749 *N* thiosulfate required were (1) 6.310, (2) 6.300, (3) 6.305, (4) 6.300, giving the normality of the HS—glutathione solution 0.002980; normality, by weight, 0.00295; by direct iodometric titration in acidified iodide solution, 0.00281.

*Iodosobenzoate and Amino Acids*—The following amino acids (in solutions or suspensions corresponding to 0.01 *M*) were found to be unaffected by *o*-iodosobenzoate in phosphate at pH 7 under the conditions described for the estimation of the substituent sulfhydryl groups of denatured albumin: tyrosine, cystine, tryptophane, proline, methionine, serine, and argi-

<sup>10</sup> Dr. Curt C. Porter kindly performed these tests.



nine. This experimentation disclosed the necessity of adding *acidified* iodide after the antecedent action of the iodoso reagent upon protein. If neutral iodide is added before acidification, the iodine formed by the oxidizing action of excess *o*-iodosobenzoate may form with tryptophane at pH 7 the wine-colored derivative; if acid is added before iodide, cystine and methionine begin to be oxidized by the acidified *o*-iodosobenzoate; difficulties are obviated by the use of *freshly acidified alkali iodide*.

Non-interference by sensitive amino acids does not guarantee lack of reactivity of *o*-iodosobenzoate with *combined* amino acids (other than cysteine) in proteins. However, there is significant increase in the degree of probability that the only reducing groups estimated are the sulphydryl.

*Estimation of Total Sulphydryl Content of Denatured Urease*—(1) Of Preparation B (Table I) each of two 0.500 ml. portions was treated with water, 1.5 ml., *N* sodium phosphate buffer of pH 7, 0.5 ml., and pure guanidine hydrochloride, 1.0 gm. The mixtures were allowed to stand at room temperature under nitrogen for  $\frac{1}{2}$  hour. To each were added 3.000 ml. of 0.002072 *N* *o*-iodosobenzoate; after 2 minutes there was added 0.5 gm. of potassium iodide dissolved in 3.0 ml. of *N* hydrochloric acid, and the iodine was titrated with 0.001826 *N* thiosulfate; thiosulfate required, 2.450, 2.490 ml.

On the assumption that 130,000 units  $\approx$  1.0 gm. of urease and that extraneous protein in Preparation B (1900 units per ml.) does not contain reducing material, the data give for the reducing equivalents ( $\approx$  residue weights of  $-\text{SH}$ ) in 21,300 gm. of urease, 4.8 and 5.0.

(2) Three 1.000 ml. portions of denatured urease of Preparation C (2815 units per ml.) were found equivalent to the following quantities of *N* *o*-iodosobenzoate: (a) 0.00504, (b) 0.00505, (c) 0.00502 ml. (With 1 ml. quantities of this potent urease, *the reproducibility in the results was excellent*.)

The data give for gm. of urease containing the equivalent of 1  $-\text{SH}$  residue weight, 4300 gm.; accordingly, there are 5 reducing equivalents ( $\approx$  residue weights of  $-\text{SH}$ ) in 21,300 gm. of urease.

For the reasons cited earlier, the number 5 probably must be considered no more than an upper limit for the total sulphydryl content, notwithstanding the recurrence of this figure in analyses of three different urease preparations.

In the estimation of the reducing capacity of 0.5 to 1.0 ml. portions of denatured urease, porphyrindin was found inferior to *o*-iodosobenzoate, particularly in respect to precision and reproducibility of results. However, the order of magnitude of the reducing equivalents found was the same; found, 4 to 4.5 equivalents per 21,300 gm. of urease.

## SUMMARY

1. Evidence of a quantitative nature is presented which bears upon the rôle of certain of the protein sulfhydryl groups in relation to the control of the activity of urease, a sulfhydryl enzyme. It has been shown that suitable treatment of urease having an activity of approximately 2500 units per ml. and 100,000 units per gm. with standard *p*-chloromercuribenzoate solution, corresponding to 2 moles of the mercuribenzoate for close to 21,300 gm. of this protein-enzyme, results in enzymatic inactivation. The inactivation is fully reversed with cysteine. Addition of the 1st mole involves no decrease in catalytic activity; addition of the 2nd is accompanied by its abolishment. Of the 2 residue weights of protein sulfhydryl which must be "removed" for completion of the inactivation, the first may be acted upon not alone by *p*-chloromercuribenzoate (or other suitable organometallic compounds) but alternatively by means of appropriate treatment with iodoacetamide, porphyrindin, or *o*-iodosobenzoate; the subsequent addition of but 1 mole of mercuribenzoate is then accompanied by reversible inactivation. These 2 sulfhydryl residue weights have been differentiated somewhat tentatively into categories, designated for convenience as (1) the *a* groupings, possibly surface-substituted and thus immediately available to the action of nitroprusside, mercaptide-forming metallic compounds, iodoacetamide, and certain oxidizing agents, and (2) the *b* groupings, still capable of rather rapid interaction with *p*- or *o*-mercuribenzoate to form, presumably, slightly dissociated mercaptide linkages, but relatively less available to the action of certain other reagents, whether for structural or other reasons. On this basis, the *b* groupings appear to be more directly concerned with the enzymatic activity. The *b* groupings are attacked by concentrated solutions of iodosobenzoate and iodoacetamide.

2. With porphyrindin and *o*-iodosobenzoate, respectively, sulfhydryl groups in considerable excess of the *a* and *b* groups have been estimated in urease, denatured in guanidine hydrochloride solution.

3. Recrystallized and dialyzed hen's egg albumin, after denaturation with guanidine hydrochloride, has been examined for its sulfhydryl content by titration with standard *o*-iodosobenzoate. The method developed gives results characterized by excellent reproducibility. The validity of this application of the method is strengthened by its utilization in the estimation of cysteine (discussed in an earlier communication) and of reduced glutathione, and further by observations that sodium *o*-iodosobenzoate, under the conditions used, fails to oxidize certain amino acids, notably tyrosine, tryptophane, serine, and methionine.

4. The results of the investigation appear to bear upon certain char-

acteristic differences in the behavior of various sulfhydryl-containing proteins and protein-enzymes, with reference particularly to divergencies in their reactivity with various alkylating and oxidizing reagents.

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# STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

## XI. HYPOPROTHROMBINEMIA IN THE RAT INDUCED BY SALICYLIC ACID\*

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The anticoagulant and hemorrhagic agent 3,3'-methylenebis(4-hydroxycoumarin) does not affect the clotting power of blood or plasma *in vitro* (1, 2). When given orally to various species of experimental animals and man, its hypoprothrombinemia-inducing action does not become detectable (even with diluted plasma) until a lapse of 12 to 24 hours (3). The onset of the hypoprothrombinemia can be advanced only a few hours by administering the anticoagulant intravenously in the form of its disodium salt.

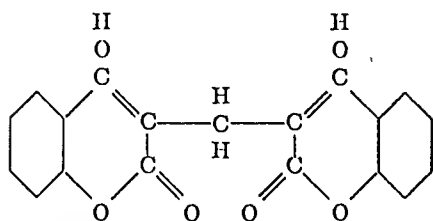
One of us (W. R. S.) has shown that, when a single dose of the anticoagulant is administered intravenously to rabbits, only traces are detectable in the blood stream by spectrographic means after a period of 20 to 24 hours (unpublished data). So it appears that, when a single dose of 3,3'-methylenebis(4-hydroxycoumarin) is injected intravenously, most of it disappears from the blood stream before the hypoprothrombinemia reaches the maximum intensity. It has already been indicated that, when massive doses were fed to dogs, some of the anticoagulant appears in the feces unchanged, but there were no indications that it was excreted in the urine (3).

One way to rationalize these observations is to assume that 3,3'-methylenebis(4-hydroxycoumarin) inhibits the formation of prothrombin by the liver and that increased prothrombin times do not become apparent until the original prothrombin level (or activity) in the blood stream has diminished. An alternative explanation is that time is required to inactivate (or qualitatively alter) the prothrombin present. A third possibility is that 3,3'-methylenebis(4-hydroxycoumarin) must undergo chemi-

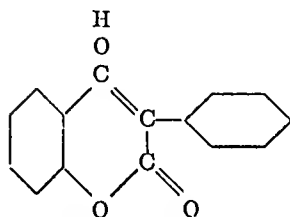
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Part of the work embodied in this paper is taken from a thesis by Dr. Ralph S. Overman presented to the Faculty of the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy, July, 1942.

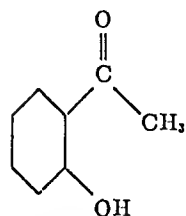
cal change in the animal body before it acts via either of the aforementioned routes.



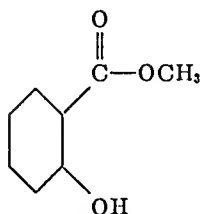
3,3'-Methylenebis(4-hydroxycoumarin)



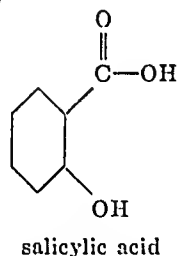
3-Phenyl-4-hydroxycoumarin



*o*-Hydroxyacetophenone



Methyl salicylate



salicylic acid

The quantitative chemical degradation of 3,3'-methylenebis(4-hydroxycoumarin) to salicylic acid was established by one of us (C. F. H.) (2, 4). Subsequently a study of the physiological activity of various analogues of 3,3'-methylenebis(4-hydroxycoumarin) and structurally related compounds revealed that only those compounds show anticoagulant action

which theoretically might yield salicylic acid or an *o*-hydroxybenzoic acid derivative on degradation.<sup>1</sup> This generalization becomes apparent from the accompanying scheme.

The purpose of this communication is to show that, when single doses of salicylic acid are administered either orally or intravenously to rats maintained on a basal artificial ration low in vitamin K, a temporary hypoprothrombinemia can be induced, comparable in all respects to that caused by 3,3'-methylenebis(4-hydroxycoumarin) (3, 5). When the rats are maintained on a stock grain ration containing vitamin K or when the basal artificial diet is supplemented with synthetic 2-methyl-1,4-naphthoquinone, the hypoprothrombinemia does not develop. It should be emphasized (a) that the prothrombin time of 12.5 per cent plasma will indicate the onset of the hypoprothrombinemia *before* changes in the prothrombin time of whole plasma are observed, (b) that the whole blood clotting times will usually be unaffected when the reduction in the prothrombin level (or activity) becomes detectable, and (c) that hemorrhage does not appear unless the salicylic acid is fed or injected continuously over a period of time. Since the change in prothrombin time reflected by the 12.5 per cent plasma is readily detected and reproducible, we have given our data only in terms of this value (3, 6).

### Methods

The methods used have all been described in previous papers of this series (5-7). It should be emphasized that the artificial ration<sup>2</sup> is low in vitamin K, while the stock grain ration<sup>3</sup> contains this dietary factor. Both rations are adequate for the growth and maintenance of rats. The animals were maintained on the appropriate basal rations a month before use (about 10 days suffice). The salicylic acid given orally was mixed into cooked corn-starch containing 2 per cent cottonseed oil. This was then mixed with the artificial ration so that the desired amount of salicylic acid was contained in 2 gm. of the final mixture. After a preliminary fasting period of 12 hours the rats were fed the salicylic acid ration mixture. 4 hours after it was consumed, access to the ration was permitted *ad libitum*. For the injection trials the desired amount of sodium salicylate dissolved in water was administered slowly into the vein of the rat's tail.

For reasons already emphasized a plasma concentration of 12.5 per

<sup>1</sup> Data obtained by Dr. M. Stahmann and others here are to be published later.

<sup>2</sup> The artificial ration comprises casein 18 parts, yeast 8, salts 4 (Wesson, L. H., *Science*, 75, 334 (1932)), Wesson oil 5, cod liver oil 2, and cerelose 63.

<sup>3</sup> The stock grain ration comprises corn 40 parts, soy bean meal 10, linseed oil meal 15, alfalfa leaf meal 3, NaCl (iodized) 1,  $\text{Ca}_3(\text{PO}_4)_2$  1, skim milk powder 20, butter fat 10.

cent was used to follow the hypoprothrombinemia (3, 6). The plasma from the rats on either ration was relatively uniform in its prothrombin level (or activity), the average prothrombin time at the 12.5 per cent concentration being 39 seconds (range 36 to 45).

#### EXPERIMENTAL

*Effect of Prolonged Ingestion of Salicylic Acid on Survival Time of Rats*—The results from many trials will be reported in a highly condensed form. Rats maintained on the stock grain ration survived a daily intake of salicylic acid over a period of 60 days at the following levels: 2.0, 10.0, and 100 mg. A mild hypoprothrombinemia at the higher level was detectable in some of the animals. The rats maintained on the artificial ration developed a severe hypoprothrombinemia in 20 days at the 100 mg. level.

TABLE I

*Effect of Single Oral Doses of Salicylic Acid on Prothrombin Time of 12.5 Per Cent Plasma from Rats Maintained on Artificial Ration*

Salicylic acid fed	No. of rats	Prothrombin time in sec. of 12.5 per cent plasma							
		Normal		16 hrs. after feeding		24 hrs. after feeding		40 hrs. after feeding	
		Average	Range	Average	Range	Average	Range	Average	Range
mg.									
10	12	39	36-44	40	38-45	49	45-52	36	35-39
25	15	39	37-44	47	44-52	50	48-55	40	37-43
100	12	39	37-45	53	49-60	65	58-70	49	45-52

When the salicylic acid intake was raised to 300 mg. per day on the artificial ration, severe hypoprothrombinemia developed in 5 days, the prothrombin time of the 12.5 per cent plasma being 75 seconds; the survival time was 10 days (average) for a group of six animals. The hemorrhagic manifestations resembling those of the sweet clover disease in cattle developed. Since the hemorrhagic condition induced by 3,3'-methylenebis(4-hydroxycoumarin) in the rat has already been fully described in a previous publication in this series (5), a complete description of the gross pathology is not necessary.

*Effect of Single Oral Doses of Salicylic Acid on Prothrombin Time of 12.5 Per Cent Plasma*—Single oral doses of salicylic acid, at the 10, 25, and 100 mg. levels, did not prolong the prothrombin time of rats maintained on the stock grain ration. In sharp contrast, the rats maintained on the artificial ration developed a temporary hypoprothrombinemia at all levels. The results with the rats maintained on the artificial ration are summarized in Table I.

*Effect of Sodium Salicylate Administered Intravenously on Prothrombin*

*Time of 12.5 Per Cent Plasma*—The results with rats maintained on the artificial ration are summarized in Table II. It should be noted that a single 25 mg. dose of sodium salicylate prolonged the average prothrombin time of the 12.5 per cent plasma from the normal of 39 seconds to 72 seconds.<sup>4</sup> The 50 mg. dose of sodium salicylate produced substantially the same degree of prolongation. Under our conditions a prothrombin time of 72 seconds of the 12.5 per cent plasma represents the degree of hypoprothrombinemia existing in rats which have developed hemorrhage after receiving 300 mg. of salicylic acid daily for 5 days while maintained on the artificial ration. It has been observed that, when the prothrombin time is prolonged by a single dose of sodium salicylate administered intravenously, no tendency to hemorrhage is indicated. This is reminiscent of our previous experience with various species of experimental animals with single large doses of 3,3'-methylenebis(4-hydroxycoumarin). This

TABLE II

*Effect of Sodium Salicylate Administered Intravenously on Prothrombin Time of 12.5 Per Cent Plasma from Rats Maintained on Artificial Ration*

Sodium salicylate injected	No. of rats	Prothrombin time in sec. of 12.5 per cent plasma					
		Normal		12 hrs. after injection		24 hrs. after injection	
		Average	Range	Average	Range	Average	Range
mg.							
5.0	10	39	37-42	39	36-41	39	36-41
25.0	30	39	35-43	72	44-95	39	35-42
50.0	12	39	37-43	76	50-100	38	36-42

observation with the methylenebiscoumarin has been substantiated by others ((3) p. 953, (8-10)).

*Effect of 2-Methyl-1,4-naphthoquinone on Hypoprothrombinemia Induced by Administration of Salicylic Acid to Rats*—The results are summarized in Table III. The protective action of the quinone is so striking that a detailed discussion of the data is not necessary. These results supply the basis for the differences in the response of the rat to salicylic acid when maintained on the stock grain ration as opposed to the artificial ration. In sum, it appears that the rats maintained on the artificial ration are receiving vitamin K at a level close to the minimum required, which makes it possible for salicylic acid to induce the hypoprothrombinemia readily (11). The utilization of vitamin K by this species had already been indicated in part through our studies with 3,3'-methylenebis(4-hydroxycoumarin) ((5) p. 600). We should also state at this point that the

<sup>4</sup> Confirmed by Dr. K. K. Chen and Mr. Charles L. Rose in the Lilly Research Laboratories, Indianapolis.



salicylic acid hypoprothrombinemia can only be induced with difficulty in those species (*i.e.*, the rabbit and dog) in which experimental avitaminosis K cannot be induced readily (12-14). However, we have had no difficulty in inducing salicylic acid hypoprothrombinemia in dogs whose livers have been injured through prolonged chloroform anesthesia (15). These dogs had been on an adequate stock diet for 30 days subsequent to the chloroform intoxication. They had exhibited normal prothrombin times for 10 days or more before the salicylic acid hypoprothrombinemia was induced. This work will be reported in detail later.

*Comparative Effect of Sodium Salicylate and 3,3'-Methylenebis(4-hydroxycoumarin) on Prothrombin Time of 12.5 Per Cent Rat Plasma*—The following observations were all made with rats maintained on the artificial ration.

TABLE III

*Effect of 2-Methyl-1,4-naphthoquinone on Hypoprothrombinemia Induced by Salicylic Acid in Rats Maintained on Artificial Ration*

Quinone and salicylic acid administered (15 rats in each group)	Prothrombin time in sec. of 12.5 per cent plasma			
	Normal		24 hrs. after administration	
	Average	Range	Average	Range
25.0 mg. salicylic acid (oral), no quinone	38	36-44	50	45-60
25.0 " " " + 1.0 mg. quinone (oral).....	39	36-43	32	30-38
			12 hrs. after administration	
25.0 mg. sodium salicylate (intravenous), no quinone.....	39	36-44	72	44-90
25.0 mg. sodium salicylate + 1.0 mg. quinone sulfonate (intravenous).....	38	35-43	34	30-44

There is a wide difference in the detectable dose between the two substances. When given orally, the action of 1.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) is readily detectable within 12 hours (increase from 39 seconds to 55) (see (5) Fig. 1), while 10.0 mg. of salicylic acid are required to effect a significant increase in the prothrombin time of the 12.5 per cent plasma. To induce a state of hypoprothrombinemia comparable to that realized with 2.5 mg. of 3,3'-methylenebis(4-hydroxycoumarin) given intravenously, approximately 50.0 mg. or more of sodium salicylate are required.

It is a well established fact that salicylic acid is distributed rapidly in the body following administration and that it has been found to enter into nearly every secretion, fluid, and organ ((16) p. 25). In fact, distribution following oral administration is so rapid that in clinical practice there is no

substantial advantage in giving it parenterally. The rapid diffusion throughout the body offers a partial explanation for the relative difference in the dosage required to produce a comparable hypoprothrombinemic effect with the more slowly eliminated 3,3'-methylenebis(4-hydroxycoumarin). The effect of 25.0 mg. of sodium salicylate given parenterally can be readily detected within 3 to 6 hours, its action being displayed more rapidly than that of a 2.5 mg. dose of 3,3'-methylenebis(4-hydroxycoumarin). The maximum hypoprothrombinemia-inducing effect from a single dose of sodium salicylate (intravenous) is realized within 12 hours,

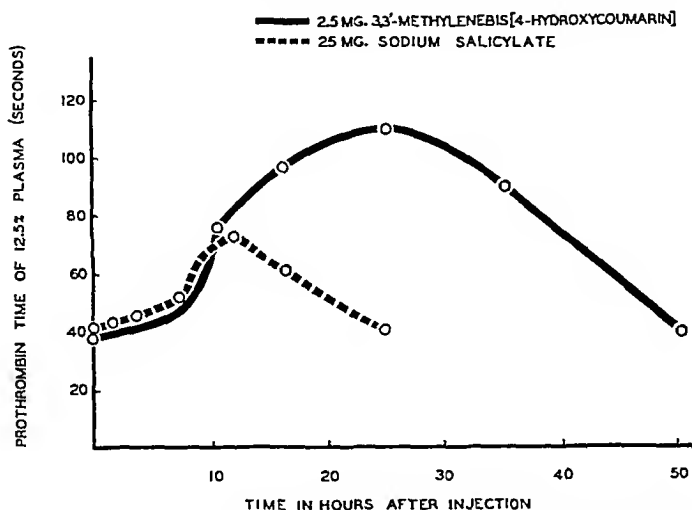


FIG. 1. The comparative effect of sodium salicylate and 3,3'-methylenebis(4-hydroxycoumarin) administered intravenously on the prothrombin time of 12.5 per cent plasma (average response of six rats).

normal prothrombin values being restored within 24 hours. In contrast, 3,3'-methylenebis(4-hydroxycoumarin) acts more slowly and the action persists over a more prolonged period (see Fig. 1).

*Proof That Prolonged Clotting Times Caused by Salicylic Acid Are Primarily Due to Induced Hypoprothrombinemia*—Evidence for the salicylic acid hypoprothrombinemia does not rest solely on the figures clocked off in our prothrombin method on 12.5 per cent plasma. We have been engaged for some time<sup>5</sup> on a comparative study of the hypoprothrombi-

<sup>5</sup> These studies were begun in 1937 by Dr. H. A. Campbell and were recently extended by Dr. Ralph S. Overman (doctoral dissertation, July, 1942) and are now being carried on by Mr. Lester D. Scheel.

nemic plasmas obtained by the following routes: (a) deep chloroform anesthesia of dogs (15), (b) administration of 3,3'-methylenebis(4-hydroxycoumarin) to rabbits, dogs, rats, and guinea pigs (3), (c) feeding sulfaguanidine to rats (17), and (d) administration of salicylic acid to rats and dogs. Since it is our hope that a complete report of this study may eventually be realized in spite of the present contingencies, we feel that no useful purpose would be served by unduly lengthening this paper with detailed data that might better be included under another title. Suffice it to state that the various plasmas exhibit substantially the same properties in so far as the prothrombin activity is concerned. The sustaining points on the salicylic acid hypoprothrombinemic plasma might be collated at this time and they are as follows:

1. A series of dilution curves was made with normal rat plasma and salicylic acid hypoprothrombinemic plasma with physiological saline as the diluting medium. These curves are substantially like those obtained with hypoprothrombinemic plasma resulting from the action of 3,3'-methylenebis(4-hydroxycoumarin) (see (6) Fig. 1, p. 8, and (3) Fig. 5, p. 951).

2. If a salicylic acid hypoprothrombinemic plasma is diluted with prothrombin-free plasma instead of the saline solution, there is no restoration of the prothrombin time over the whole dilution range. The prothrombin-free plasma was prepared by the method of Mellanby (18).

3. If a salicylic acid hypoprothrombinemic plasma is reconstituted with normal rat plasma, the normal prothrombin times are restored. This holds over a wide range of dilutions—a small quantity of normal plasma (or prothrombin preparation) has the capacity to restore the prothrombin time of appreciable quantities of hypoprothrombinemic plasma.

4. If a beef prothrombin preparation<sup>6</sup> dissolved in saline solution obtained by the method of Seegers (19) is added to salicylic acid hypoprothrombinemic plasma, the normal prothrombin times can be restored over the whole range of a dilution series.

5. Finally, if salicylic acid hypoprothrombinemia is induced in a rat, the prolonged prothrombin times can be reduced rapidly by injecting a prothrombin preparation dissolved in saline solution.

#### DISCUSSION

The thesis that 3,3'-methylenebis(4-hydroxycoumarin) is metabolized in the body to salicylic acid and thereby exerts its hypoprothrombinemia-inducing effect obviously cannot be accepted until salicylic acid, a derivative of salicylic acid, or some definitive degradation product arising from

<sup>6</sup> We are indebted to Messrs. Carl Pfeiffer and Daniel A. McGinty, Research Laboratories, Parke, Davis and Company, Detroit, for supplying some of the prothrombin preparations used in this study.

either has been isolated from the tissue. We are engaged in the pursuit of this objective. Sustaining evidence for the thesis is that the isomeric *m*- and *p*-hydroxybenzoic acids, as well as benzoic acid, do not induce the hypoprothrombinemia when administered intravenously.

Some 50 years ago the German pharmacologist Binz emphasized in his lectures (which also became available in the English language) that, when salicylic acid is given to certain individuals suffering from rheumatic fever,<sup>7</sup> it causes hemorrhage from the mucous membranes (20).<sup>8</sup> Binz left the mechanism unexplained, but advised caution in the use of the drug (21). Subsequently some investigators held that the hemorrhagic manifestations observed by Binz were perhaps due to the febrile condition and not to direct action of the salicylic acid (16). In 1926 Wetzel and Nourse (22) reviewed all the cases of oil of wintergreen (methyl salicylate) poisoning previously reported and concluded that in addition to vascular changes the most general lesion is hemorrhage. In 1939 Sir Arthur Hurst of Guy's Hospital, London, in a report on aspirin hematemesis (23), wrote that the wide-spread use and abuse of aspirin<sup>9</sup> makes the recognition of its toxic effects of more than academic interest and accumulating evidence of its power to produce severe gastric hemorrhage necessitates the consideration of the possible rôle of this drug in all cases in which the more common causes of bleeding can be excluded.

To our knowledge the extensive literature on the pharmacology and therapeutic use of salicylates does not indicate that salicylic acid can affect the blood coagulation mechanism *per se* (16). Furthermore, the literature does not contain reports that salicylic acid (salicylates) acts as an anti-coagulant *in vitro*. Extensive trials by us with whole blood and plasma from various species of mammals, which need not be given here, also indicate that salicylic acid or sodium salicylate has no effect on the clotting powers of normal blood or plasma *in vitro*.

In view of the current belief (13, 24-26) that in adult man dietary deficiencies of vitamin K severe enough to produce clinical hypoprothrombinemia are rarely encountered, we would have hesitated to draw on the aforementioned medical literature if it were not for the fact that our findings on hypoprothrombinemia induced by salicylic acid in rats have already been put to test by two independent clinical investigators.

<sup>7</sup> The amount prescribed in acute rheumatic fever is much larger than that employed for any other purpose. In fact the dosage is colossal (see (16)).

<sup>8</sup> Binz indicated that in females where this tendency exists the use of salicylates may occasion frequent or excessive menstruations or if the patient is pregnant may cause miscarriage or premature birth. Prior to Binz, French physicians had asserted that one of the direct effects of salicylic acid is to cause abortions (the literature is cited by Binz (20)).

<sup>9</sup> See the editorial comment in *The Journal of the American Medical Association* (115, 1199 (1940)) and the annotation in *The Lancet* (1, 1091 (1940)).

The basic observations on rats became known to us for the first time in June, 1941. We revealed the findings and our matured interpretations to Dr. Shepard Shapiro, M.D., Welfare Hospital, New York University Division III, New York, and Dr. O. O. Meyer, M.D., Wisconsin General Hospital, Madison, in early August, 1942.<sup>10</sup> Both clinicians have reported that our observations are reproducible in man *even when the diets are not restricted*. The clinical reports will appear elsewhere.

We do not feel that speculation on the interrelationship of 2-methyl-1,4-naphthoquinone metabolism and the salicylic acid hypoprothrombinemia is warranted at present, since we know so little about how the quinones function in prothrombin synthesis (12, 13, 27). Several attractive alternatives should be obvious to anyone after a little meditation. As we see it, the work reported herewith has in a sense raised more questions than it has answered. But it is our hope that it might lead to new approaches to the poorly understood beneficial therapeutic action of salicylates<sup>11</sup> (16, 28-30), in addition to offering at least a partial explanation for one of the untoward effects that they might induce under certain conditions (20, 23, 31).

A special acknowledgment is due to my eldest brother, Dr. George K. Link, Professor of Botany and Plant Pathology, University of Chicago, whose critical faculty and broad philosophic outlook on biological phenomena have been a great help to me in connection with our studies on the hemorrhagic sweet clover disease (K. P. L.).

#### SUMMARY

1. It is shown that single doses of salicylic acid induce a temporary hypoprothrombinemia in rats when the rats are maintained on a ration low in vitamin K.

<sup>10</sup> Our findings were also revealed to Dr. K. K. Chen, M.D., Ph.D., at the Lilly Research Laboratories, Indianapolis, who with the assistance of Mr. Charles L. Rose kindly checked the results on rats. In a letter dated September 18 he stated, "The hypoprothrombinemia-inducing effect of salicylic acid which you have observed can be easily substantiated." The responses obtained by Messrs. Chen and Rose with 25 mg. of sodium salicylate per kilo (intravenous administration) practically duplicate the figures in Table II. We wish to thank them for their generous and prompt cooperation.

<sup>11</sup> It is generally held that salicylates exert their typical effects by virtue of the salicylic acid content, variations in solubility and propensity to cause local irritation being the main factors which determine the degree of usefulness. The efficacy of the different salicylates depends in part on the amounts of salicylic acid liberated in the body, but not entirely on this factor. Thus it is reasonable to assume that the hypoprothrombinemia-inducing effects of the various salicylate drugs would vary. We already have evidence pointing in this direction.

2. The salicylic acid hypoprothrombinemia does not develop when rats are maintained on a diet containing vitamin K.

3. The synthetic quinone (2-methyl-1,4-naphthoquinone or the water-soluble sulfonate) protects the rat against the hypoprothrombinemia-inducing action of salicylic acid.

4. The parallelism between the hypoprothrombinemia and hemorrhage induced by 3,3'-methylenebis(4-hydroxycoumarin), the causative agent of the hemorrhagic sweet clover disease, and salicylic acid is indicated.

5. The bearing of these findings on some of the long standing contraindications reported in the therapeutic use of salicylates in medicine (hemorrhage) is discussed.

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# HYDROGENASE AND SYMBIOTIC NITROGEN FIXATION\*

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In 1941 Phelps and Wilson (1) demonstrated the presence of hydrogenase in *Azotobacter vinelandii* and suggested that it might be significant for nitrogen fixation by this species. This proposal, originally based on the fact that molecular hydrogen is a specific inhibitor for nitrogen fixation by this organism, has been supported by additional investigations (2).<sup>1</sup> Since H<sub>2</sub> also inhibits nitrogen fixation by the symbiotic system of leguminous plants inoculated with the root nodule bacteria (*Rhizobium* sp.), it was thought probable that these species likewise might possess hydrogenase. Tests of pure cultures grown on laboratory media were negative, but suspensions of the organism prepared directly from pea nodules by the method of Thorne and Burris (3) apparently contained the enzyme. This was not true, however, of cultures from nodules of the soy bean (1). Before these erratic results could be resolved, the 1941 growing season ended, and investigations on the bacteria from nodules of leguminous plants had to be discontinued until the following year. This paper describes our more recent tests on these organisms.

## Methods

Our original observations were based on a modified Thunberg method in which the rate of reduction of methylene blue in the presence and absence of H<sub>2</sub> was measured. The control was a tube which had been evacuated by a water pump. At times an inert gas was added to this control: N<sub>2</sub>, He, or A. When this was done, the tube was flushed once with the gas which was passed through a tower of alkaline pyrogallol to remove O<sub>2</sub> impurities. Although this method is quite satisfactory for suspensions which possess a powerful hydrogenase, such as *Azotobacter*, it has several potential sources of errors. These include (a) failure to evacuate to the same degree in control and experimental tubes, (b) failure to remove the last traces of oxygen from the displacing gases. Because of our erratic results with nodule suspensions it seems likely that, if a hydrogenase is present in such cultures, its activity will be feeble; therefore, demonstration will require more precise

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<sup>1</sup> Unpublished data from this laboratory.



and reliable procedures than the one previously used. We have employed three improved methods for detecting hydrogenase.

*Modified Thunberg Method*—1 ml. of bacterial suspension is placed in the hollow stopper of the Thunberg tube described by Tam and Wilson (4). The tube itself contains 1 ml. of 0.00005 M methylene blue, 3 ml. of M/15 phosphate buffer (pH 7.5), and 5 ml. of distilled water. It is evacuated with a Hyvac pump (rated final vacuum of  $10^{-3}$  mm.). The last traces of air are swept out by the vaporization of water which is condensed in a dry ice trap. The cylinder gases are freed of oxygen by slow passage over copper turnings heated to about  $800^{\circ}$ . After passing through a dry ice trap the gases are collected in glass bulbs evacuated to  $10^{-3}$  mm. of Hg. A gas is transferred from the storage bulb to the evacuated Thunberg tube by means of a Urey pump (5).

After temperature equilibration at  $35^{\circ}$ , the suspension is mixed with the contents of the tube, and the reduction of the methylene blue measured in a Coleman universal spectrophotometer at a wave-length of 660 m $\mu$ . Readings are made every 5 minutes; when  $\log I_0/I$  is plotted against time, a straight line results whose slope measures the rate of reduction of the dye.  $I_0$  is the final galvanometer reading after complete reduction of dye with sodium hydrosulfite;  $I$  is the reading at any time,  $t$ .

*Modified Thunberg-Warburg Method*—The foregoing method was modified by carrying out the reduction in the Warburg micro respirometer and measuring the rate of  $H_2$  uptake instead of the reduction of methylene blue. In a flask whose side arm contains 1 ml. of 0.002 M methylene blue are placed 1 ml. of bacterial suspension and 1 ml. of M/15 phosphate buffer of pH 7.5; if an inhibitor is to be added, 0.5 ml. of a double strength bacterial suspension and 0.5 ml. of inhibitor solution are used. KOH is placed in the center well to absorb any anaerobic  $CO_2$  produced. After temperature equilibration at  $35^{\circ}$ , the manometers are observed for 10 to 20 minutes to check that no "endogenous" gas uptake occurs, then the methylene blue is tipped in, and the uptake of gas measured. After 20 to 30 minutes the reduction of the methylene blue is complete, and no further change in the manometer occurs.

*Knallgas Reaction*—While investigating various possibilities for more precise methods of measuring hydrogenase activity, we found that cultures of *Azotobacter* readily use  $O_2$  as the  $H_2$  acceptor. A method was then developed based on this *Knallgas* reaction (6). Although the original method was satisfactory for *Azotobacter*, this would not assure that it would be with nodular suspensions which possess an unusually high endogenous respiration. Accordingly, we sought for differential inhibitors which would depress  $O_2$  uptake due to respiration more than that due to oxidation of  $H_2$ . Three such inhibitors were found (7), two of which (iodoacetate and hydroxylamine) have been used extensively in this work.

Before use on nodular suspensions all methods were thoroughly tested with *Azotobacter*, and found to be both sensitive and reliable. The reduction of methylene blue in the modified Thunberg method no longer showed an initial lag (1), indicating that the last traces of  $O_2$  had been removed; even with quite dilute suspensions of *Azotobacter* pronounced hydrogenase activity was evident. In the Thunberg-Warburg method no change in the manometer occurred (after thermobarometric correction) until the methylene blue was added. With *Azotobacter*, gas equivalent to the  $H_2$  required to reduce the added dye disappeared, after which the readings once more became stable. The precision and accuracy of the method based on  $H_2$ - $O_2$  uptake have been discussed in previous reports (6, 7).

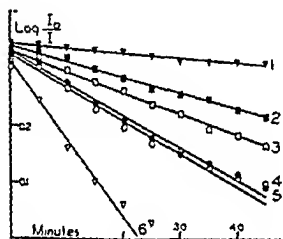


FIG. 1

FIG. 1. Test for hydrogenase in suspensions from nodules by the modified Thunberg technique. Curve 1, He, *Azotobacter*; Curve 2, He, soy bean; Curve 3,  $H_2$ , soy bean; Curve 4, He, cow-pea; Curve 5,  $H_2$ , cow-pea; Curve 6,  $H_2$ , *Azotobacter*.

FIG. 2. Test for uptake of  $H_2$  by nodular suspensions in presence of methylene blue. Zero time indicates the point at which the dye was tipped in. Curves 1,  $H_2$ , *Azotobacter*; Curves 2, He, *Azotobacter*; Curve 3,  $H_2$ , cow-pea; Curve 4, He, cow-pea; Curve 5,  $H_2$ , soy bean; Curve 6, He, soy bean.

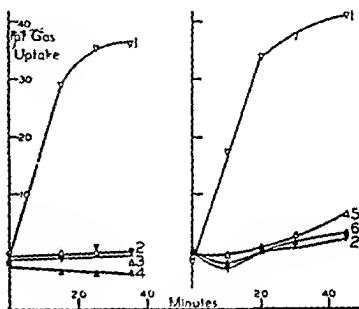


FIG. 2

#### EXPERIMENTAL

Results from typical experiments in which the improved methods were used are shown in Figs. 1 to 4. Since suspensions from nodules of the cow-pea and soy bean reduce methylene blue as rapidly in a helium atmosphere as in hydrogen (Fig. 1), it is concluded that the reduction arises from dehydrogenases acting on endogenous substrates. With *Azotobacter* reduction is much more rapid in  $H_2$ . In this particular trial, the rate of reduction by the soy bean suspension is somewhat more rapid in  $H_2$ , but it hardly exceeds experimental error and was not observed in other experiments.

Consideration of the lines in Fig. 1 suggests that the Thunberg method may not be too suitable for demonstration of hydrogenase in nodular sus-

pensions, since reduction of the dye by dehydrogenases which use endogenous donors may obscure the reduction by  $H_2$ . This confusion of activity is eliminated in the Warburg-Thunberg method in which the  $H_2$

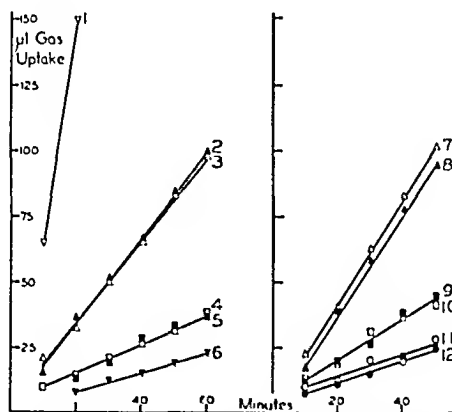


FIG. 3. Test for *Knallgas* reaction in nitrogen-fixing bacteria. Left, suspension from cow-pea nodules; right, suspension from soy bean nodules. Curve 1, *Azotobacter* in  $H_2-O_2$ ; Curve 6, *Azotobacter* in  $He-O_2$ ; Curves 3 and 7,  $H_2-O_2$  mixture; Curves 2 and 8,  $He-O_2$  mixture; Curves 4 and 9,  $H_2-O_2$  plus 0.01 M iodoacetate; Curves 5 and 10,  $He-O_2$  plus 0.01 M iodoacetate; Curve 11,  $H_2-O_2$  plus 0.002 M hydroxylamine; Curve 12,  $He-O_2$  plus 0.002 M hydroxylamine. The values for the inhibitors refer to the final concentration.

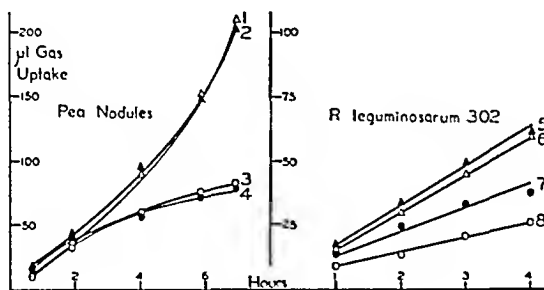


FIG. 4. Test for *Knallgas* reaction in pure and nodular cultures. Curves 1 and 6,  $H_2-O_2$ ; Curves 2 and 5,  $He-O_2$ ; Curves 3 and 8,  $H_2-O_2$  plus 0.002 M hydroxylamine; Curves 4 and 7,  $He-O_2$  plus 0.002 M hydroxylamine.

used to reduce the dye is measured directly. No evidence of uptake of  $H_2$  by nodular suspensions was obtained with this method (Fig. 2). The small changes in the manometers were independent of the presence of  $H_2$  or  $He$  and can be ascribed to experimental error. When *Azotobacter* was used,

however, the uptake of  $H_2$  approached the theoretical (44 microliters) necessary for reduction of the methylene blue.

The data of Figs. 3 and 4 are typical of a large number of trials in which the *Knallgas* reaction was employed for detection of hydrogenase. Curves 1 and 6 (Fig. 3) illustrate its use with the hydrogenase in *Azotobacter*. The absence of the enzyme in nodular suspensions from soy bean or cow-pea is demonstrated by the following: (a) the uptake of gas is as rapid in an  $He-O_2$  mixture as in an  $H_2-O_2$  mixture; (b) the same is true in the presence of inhibitors such as iodoacetate and hydroxylamine which exert a differential effect on oxidation of substrate and hydrogen (7). Likewise, no evidence was obtained for the existence of hydrogenase in either the pure culture or a nodular suspension of the pea organism (Fig. 4).

It is emphasized that the data in Figs. 1 to 4 have been confirmed by similar results from a large number of experiments completed during the past growing season. In addition to trials which were essentially replication of those selected for illustration, several variations were introduced in the standard procedures outlined under "Methods." These included (a) variation in the  $pO_2$  used in the *Knallgas* reaction from 0.01 to 0.20 atmosphere, (b) use of both intact and crushed whole nodules instead of the suspension of bacteria from the nodule, (c) preliminary incubation of the suspension (or nodules) in pure  $H_2$ , (d) variation in the age and variety of the plant, (e) change in the concentration of suspension used—bacterial nitrogen varied from 0.5 to 2.0 mg. per ml. In nearly all trials the absence of a hydrogenase in the root nodule bacteria was indicated. Occasionally, results suggesting weak hydrogenase activity were obtained, but these were neither numerous nor consistent.

#### DISCUSSION

The results from these experiments make it extremely doubtful that the root nodule bacteria, either in pure culture or in association with the plant, contain hydrogenase. If this is true, a significant difference in the symbiotic nitrogen fixation system and that in the free living *Azotobacter* is suggested. The latter possesses a powerful hydrogenase which appears to be associated with its nitrogen-fixing activity (2).<sup>1</sup> At this time, however, we cannot definitely conclude that nitrogen fixation and hydrogenase are not associated in the symbiotic system. Although both pure culture and nodular suspension apparently lack hydrogenase, they also are unable to fix free nitrogen. Using the extremely sensitive nitrogen isotope method, we have been unable to detect fixation by such cultures.<sup>1</sup> Intact nodules, however, frequently assimilate detectable quantities of free  $N^{15}$ . It was this fact that led us to test nodules rather than the bacteria.

With such heterogeneous material as whole nodules the reliability of the hydrogenase measurements is greatly reduced. Because of the rapid reduction of the dye by dehydrogenases the methylene blue techniques are almost useless. In applying the method based on the *Knallgas* reaction, we weighed 1 gm. of freshly excised nodules into the Warburg flask; after the test was completed the contents of the flask were washed into a Kjeldahl flask and the total nitrogen determined. The variation among samples in total nitrogen content indicated that the quantities of active material present in the flasks were far from equal; hence uniformity in gas uptake could not be expected. Even when the results are expressed as activity per mg. of nitrogen, the variation between duplicates was generally so great that a weak hydrogenase would be obscured. The sensitivity of the method was likewise decreased by the large endogenous respiration, part of which apparently was not greatly influenced by iodoacetate and hydroxylamine. Since even the modified methods are not particularly adapted for detection of weak activity in such heterogeneous material, a better approach might be to use heavy hydrogen, just as we have used heavy nitrogen to detect nitrogenase activity.

Finally, our experience with plant cultures grown in the presence of  $H_2$  should be mentioned. In these experiments (8) ample fixation occurred even though  $H_2$  did inhibit the reaction. If the nitrogen-fixing system in the intact plant were associated with as powerful a hydrogenase as is possessed by *Azotobacter*, one would expect the disappearance of a detectable quantity of gas through operation of the *Knallgas* reaction. Although this may have occurred on a small scale (comparable with the quantity of  $N_2$  fixed), it never approached that observed when organisms known to contain a hydrogenase were present (9).

#### SUMMARY

Three modified procedures designed to improve the sensitivity of the methods for detection of hydrogenase in bacteria have been developed and applied to nitrogen-fixing organisms. In one, the rate of reduction of methylene blue is determined in a spectrophotometer; special care is taken to insure that the gases used are free of oxygen. Another combines the standard Thunberg method with the Warburg manometric procedure, so that the  $H_2$  which disappears in the reduction of methylene blue is measured instead of the time of reduction. The third measures the disappearance of gas in an  $H_2$ - $O_2$  mixture; a modification of this method includes use of differential inhibitors which selectively reduce the respiratory activity.

The reliability and sensitivity of the methods were demonstrated with *Azotobacter* which contains an active hydrogenase. Tests for this enzyme in suspensions of bacteria from nodules of pea, soy bean, and cow-pea

plants were then made. Excised whole nodules were also tested, although the methods are not particularly suited for such heterogeneous material.

The root nodule bacteria apparently do not contain hydrogenase. The relation of this finding to symbiotic and asymbiotic nitrogen fixation is discussed.

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## LETTERS TO THE EDITORS

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### INFLUENCE OF BUFFER AND GLUCOSE IN THE LACTOBACILLUS CASEI ASSAY FOR PANTOTHENIC ACID

Sirs:

Clarke *et al.*<sup>1</sup> have recently reported the formation of 20 to 23 ml. of 0.1 N acid in the *Lactobacillus casei* assay for pantothenic acid as compared to the usual maximum of 10 to 12 ml. The higher values were obtained when 400 mg. or more of rice polishings concentrate were added to 10 ml. of medium. Similar results have also been obtained with *Lactobacillus casei* in riboflavin assays of extracts of whole wheat flour, wheat bran, and rice bran.<sup>2</sup> We should like to report high acid formation in pantothenic acid assays obtained simply by increasing the amounts of glucose and sodium acetate in the assay medium. The findings are, in general, also applicable to the riboflavin assay.

It is known that a low pH as a result of lactic acid accumulation may be a limiting factor in this fermentation and that a marked increase in acid production occurs if the cultures are adequately buffered or neutralized during the fermentation.<sup>3</sup> We find that if the pantothenic acid assay medium of Pennington *et al.*<sup>4</sup> is carefully prepared to contain 0.6 per cent sodium acetate as required, an increase in glucose from 1 to 2 per cent raises the maximum amount of acid formed in 10 ml. of medium from 10.5 to 14.5 ml. in the presence of 1  $\gamma$  of pantothenic acid. 15 ml. of acid are formed with 3 per cent glucose.

If 2 per cent glucose is used, an increase in concentration of the sodium acetate buffer from the customary 0.6 per cent to 1.2 and 1.8 per cent raises the amount of 0.1 N acid formed in 10 ml. of medium from 15 to 18.5 and 19.3 ml., respectively. The additional acetate raises the buffering capacity of the assay medium as measured by potentiometric titrations with hydrochloric acid. The ratio of the amount of 1.17 N HCl required to bring the media containing 0.6, 1.2, and 1.8 per cent sodium acetate, respectively, to pH 4.0 (approximately the final pH of the fermentation)

<sup>1</sup> Clarke, M. F., Lechycza, M., and Light, A. E., *J. Biol. Chem.*, **142**, 957 (1942).

<sup>2</sup> Wegner, M. I., Kemmerer, A. R., and Fraps, G. S., *J. Biol. Chem.*, **144**, 731 (1942).

<sup>3</sup> Longworth, A. G., and MacInnes, D. A., *J. Bact.*, **32**, 567 (1936). Tatum, E. L., and Peterson, W. H., *Ind. and Eng. Chem.*, **27**, 1493 (1935).

<sup>4</sup> Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).



is 1:1.5:2.<sup>5</sup> With 1 per cent glucose, the additional acetate does not increase acid formation.

Results similar to those with acetate can be obtained by periodically neutralizing the cultures with sodium hydroxide. As much as 32 ml. of 0.1 N acid is formed in 10 ml. of medium containing 3 per cent glucose.

It is, therefore, evident that the assay medium contains sufficient nutrients and growth factors to permit much larger amounts of acid to be formed than the maximum normally obtained, provided adequate amounts of glucose and buffer are supplied. These results raise the question as to what extent the sugar content and buffering properties of extracts of rice polishings, wheat bran, etc., are responsible for the high titration values obtained with such materials. This problem is under investigation.

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<sup>5</sup> We are indebted to Dr. N. R. Trenner and Mr. W. R. Reiss of Merck and Company, Inc., for these titrations.

# MICROBIOLOGICAL ASSAYS FOR *p*-AMINOBENZOIC ACID

Sirs:

We have developed in this laboratory a microbiological assay for *p*-aminobenzoic acid, using a mutant strain of *Neurospora crassa*<sup>1</sup> very generously supplied to us by Dr. G. W. Beadle of Stanford University. Application of this method, based on studies by Lampen, Underkoffler, and Peterson,<sup>2</sup> to aqueous extracts of various natural substances yields results which suggest that the assay procedure of Landy and Dicken<sup>3</sup> responds to

TABLE I

Substances	<i>p</i> -Aminobenzoic acid	
	Hot water extract	Acid hydrolysis
	$\gamma$ per gm. moist tissue	$\gamma$ per gm. moist tissue
Beef liver.....	<0.1	2.5
Spinach.....	0.12	0.6
Oats (seed).....	0.13	0.5
Mushrooms.....	0.5	1.3
Fresh yeast....	3.6	4.0

TABLE II

	H <sub>2</sub> SO <sub>4</sub>	<i>p</i> -Aminobenzoic acid
	<i>N</i>	$\gamma$ per gm.
1 hr. autoclaving, 15 lbs.	0.1	0.9
	1.0	1.2
	6.0	2.2
	12.0	2.0

only a fraction of the total amount of *p*-aminobenzoic acid obtainable after acid or alkaline hydrolysis. We find, moreover, that enzymatic hydrolysis<sup>4</sup> or autolysis<sup>5</sup> is not always sufficient to lead to the maximum effect. The increased yield obtainable by heating with 6 *N* H<sub>2</sub>SO<sub>4</sub> for 1 hour in the autoclave at 115.5° is demonstrated by the examples in Table I.

<sup>1</sup> Tatum, E. L., and Beadle, G. W., *Proc. Nat. Acad. Sc.*, 28, 234 (1942).

<sup>2</sup> Lampen, J. O., Underkoffler, L. A., and Peterson, W. H., *J. Biol. Chem.*, 146, 277 (1942).

<sup>3</sup> Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 146, 109 (1942).

<sup>4</sup> Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *Univ. Texas Pub.* 4237 (1942).

<sup>5</sup> Blanchard, K. C., *J. Biol. Chem.*, 140, 919 (1941).

The effect of acid concentration on the yield of the vitamin from beef liver is shown in Table II.

Recently in this laboratory Dr. D. E. Pennington has developed an extremely sensitive microbiological assay method for *p*-aminobenzoic acid, using a lactic acid bacterium as the test organism. A comparison of assay results by this and the *Neurospora* method is in progress and complete details will be published in the near future.

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# SUBSTRATE SPECIFICITY OF AMINE OXIDASE

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(Received for publication, November 2, 1942)

The tyramine oxidase activity of liver extracts found by Hare (1), the aliphatic amine oxidase activity of brain, kidney, and liver extracts observed by Pugh and Quastel (2), and the adrenalin oxidase activity of similar extracts noted by Blaschko, Richter, and Schlossmann (3) were brought under a common enzyme view-point by the latter authors. They were able to show (4) that extracts of brain, intestine, kidney, and liver from a number of mammals or representatives of the birds, reptiles, amphibians, and fishes all acted to absorb oxygen in the presence of several amine substrates. Hare (1) had shown that tyramine and phenethylamine form ammonia in the course of such oxidations, and Richter (5) showed that an ethylamino and a dimethylamino compound, as well as a number of methylamino and amino compounds, all yield the corresponding alkylamines or ammonia in the enzymic oxidation.

The conclusion that the demonstrated variety of such enzymic activity can be ascribed to the presence of only a single type of amine oxidase was dependent in large part on observations that the relative activities of a preparation from one source on a series of substrates bear some relation to the relative activities exhibited by a preparation from another source. Further evidence depended upon the action of certain amines as inhibitors and apparent competition between substrates when two oxidizable substrates are present in the system. The degree to which the relative activities of different enzyme preparations were constant in a series of substrates was not good in the data reported, and the fact that Hare (1) had not been able to note activity of the liver preparations she used upon adrenalin as the substrate appeared to require special explanations.

After the present work was begun, it became obvious that relative to activity based on use of tyramine as substrate activity differences are notable on other substrates with liver extracts derived from different animal species. It appeared very desirable to attempt purification of the crude extracts used to see whether during the process changes would result in the relative activity when tested on a series of substrates. Another object of purification was to prepare material suitable for trial injection into

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animals made hypertensive, an experiment which was carried out in idea by Schroeder (6) but with doubtful adequate activity of the enzyme used.

The degree of purification achieved after very considerable work was not very great, but an effective and rapid separation from readily dialyzable substances and materials that are soluble at pH 6 was developed. The activity of such partially purified preparations was determined with considerable precision on a number of substrates to determine the effect on the oxidative reaction of small but important changes in the structure of aliphatic, phenylaliphatic, and substituted phenylaliphatic amine substrates. Such observations form a basis for consideration of the deamination of these various amines by the liver when they naturally occur or are introduced in the body, an idea that has been partially developed for some phenylaliphatic amines by Beyer and Lee (7). The kinetic equilibrium constants for amine oxidase and its amine substrates here reported may be expected to form bases for ideas of the combination of these same amines with physiologically active receptor mechanisms.

*Species Variance of Amine Oxidase*—Crude enzyme preparations were made by grinding livers of freshly killed animals with 4 ml. per gm. of 0.2 M phosphate buffer, pH 7.0, then screening to remove fiber, and centrifuging in a supercentrifuge. The test system consisted of 2.0 ml. of the enzyme solution, 0.3 ml. of 0.1 M sodium cyanide, and 0.2 ml. of a 0.1 M solution of amine salt (chloride or sulfate). Oxygen uptake was measured at 30° with the usual Warburg technique, with readings every 5 minutes. From plots of the data, maximum rates of oxygen uptake were determined and calculated into terms of per cent based on the initial rate of oxygen uptake of phenethylamine. The data of Table I are averages of two sets of experiments for each animal species.

The extent to which the data of Table I are comparable to the data given by Blaschko, Richter, and Schlossmann (4) for certain of these substrates when acted on by guinea pig or other liver preparation is impossible to determine. The observations here are corrected for the oxygen uptake of the blank, which was very small in all cases owing to working promptly with fresh extracts only. Aside from minor differences in the pH and temperature of observation, wholly different comparative values may result with certain types of substrate by taking only initial rates as the basis of observation instead of periods of as long as 1 hour. Most notably with the aliphatic amines, the rate of oxygen uptake decreases with time. With the substrate concentrations used, the falling off of rate with any of the substrates appears unrelated to disappearance of substrate or of enzyme, and is probably an inhibition of enzyme by reaction products, as found by Hare (1). Whatever the cause, it is apparent that maximum rates of oxidation are most likely to give a clearer picture of the substrate specificity (see Fig. 1).

*Purification of Amine Oxidase*—Preparation of a cell-free solution of the enzyme from rabbit liver extract by adsorption on kaolin and elution of the product was mentioned by Hare (1). Kohn (8) found the enzyme to be precipitated from pig liver extract at a reaction acid to methyl red and the insoluble material with which it was associated to be resuspendable at pH 7 to 8. Amine oxidase activity has only been observed in the present

TABLE I  
*Liver Extract Oxidations; Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine*

At 30° with 0.008 M amine substrates in phosphate buffer, pH 7.0.

	Rabbit	Guinea pig	Cat	Cattle
Ethylamine.....	0	0	20	5
Butylamine.....	50	100	90	110
Amylamine.....	110	140	110	85
Hexylamine.....	120	80	90	85
Heptylamine.....	130	70	100	85
Benzylamine.....	30	10	100	130
Phenethylamine.....	100	100	100	100
Phenpropylamine.....	110	30	90	105
Phenethanolamine.....	30	30	55	5
Phenethylmethylamine.....	105	120	105	65
3-Hydroxyphenethylamine.....	70	190	110	95
4-Hydroxyphenethylamine (tyramine)....	90	200	120	130
4-Hydroxyphenethylmethylamine.....	65	160	80	85
4-Hydroxyphenethanolmethylamine (syn- ephrine).....	25	70	40	5
4-Hydroxyphenethyldimethylamine (hor- denine).....	30	10	45	80
3,4-Dihydroxyphenethylamine (hydroxy- tyramine).....	65	200	80	85
3,4-Dihydroxyphenethylmethylamine (epi- nine).....	65	180	30	65
3,4-Dihydroxyphenethanolamine (arte- renol).....	25	40	20	5
3,4-Dihydroxyphenethanolmethylamine (dl- epinephrine).....	15	40	20	5
1-3,4-Dihydroxyphenethanolmethylamine (epinephrine).....	25	40	20	5

work in turbid "solutions," and in general the activity and turbidity of freshly prepared extracts seemed to be proportional. With salts present, the amine oxidase activity cannot be centrifuged out if the pH is 7 or higher, and only partially at lower pH values. If salts are removed by dialysis and the pH adjusted to 6.0, a flocculent precipitate separates that contains almost all of the amine oxidase activity present. Washing this

precipitate with water adjusted to pH 6.0 removes a considerable amount of inert material with only a small loss of enzyme activity; then the precipitate can be redispersed by addition of salts or adjustment to pH 7 or higher so that it cannot be removed by centrifugation in a Sharples machine. Storage of the precipitate obtained at pH 6.0 at that acidity is apt to result in a decrease in "solubility" in salt solutions or at higher pH values, but without considerable change in activity, showing that activity is observable with definitely insoluble particles that may be removed by centrifugation or filtration.

The marked instability of amine oxidase outside of the pH range of 5.5 to 8.5 greatly limits the purification procedures that may be used. Addition of but 30 per cent alcohol completely inactivates the enzyme in a short

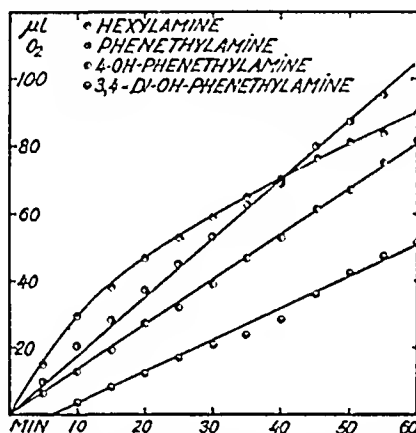


FIG. 1. Oxygen uptake in microliters against time in minutes for different substrates. At 30° with 2.0 ml. of 1:4 rabbit liver extract with 0.2 M phosphate buffer, pH 7.0, 0.3 ml. of 0.1 M sodium cyanide, and 0.2 ml. of 0.1 M amine salt solution.

time, and while precipitation of the activity may also be accomplished with addition of ammonium sulfate to one-third saturation, such salting-out is not a useful purification procedure. Such salting-out results in a considerable loss (25 to 40 per cent) of activity and does not offer any advantage over isoelectric precipitation, particularly since the high density of the salting-out solution makes difficult the centrifugation of the precipitate. Procedures involving adsorption of the activity on such agents as kaolin and tricalcium phosphate were tried under various conditions, but were not practicable because of unsatisfactory recovery of the activity from the adsorbates.

Most of the purification work from which the above conclusions were drawn was carried out with cattle liver extracts made by grinding the fresh

tissue with some ice water in a Waring blender at high speed, then making the mixture to 500 volumes per cent of the tissue weight. For later work, because of the importance of using fresh tissue for each preparation, rabbit liver extracts were chiefly used and the work reported in this paper without mention of source was carried out only with enzyme preparations from rabbit liver.

As a standard in the purification work, a unit of amine oxidase activity was taken as the amount of enzyme which will catalyze the uptake of 1 microliter of  $O_2$  per minute at  $30^\circ$  and pH 7.0 in the presence of 0.008 M tyramine. The protein content of the preparations was readily determined by pipetting 10 ml. into 20 ml. of 20 per cent trichloroacetic acid, adding 100 mg. of diatomaceous earth filter aid, filtering after 10 minutes, washing the precipitate with water, then ether, and drying at  $80^\circ$  before weighing. The stability of amine oxidase preparations is fair when kept at pH 7 in a refrigerator at  $0-5^\circ$ , and only small losses in activity were usually observed during as much as a week of storage, but an increasing ability of the preparation to absorb oxygen without any substrate addition may become considerable after only a few days and no data are reported for preparations over 4 days old. The change in the preparation on storage appears to be due to other enzymes, as the addition of 1:10,000 to 1:20,000 phenylmercuric acetate served to insure sterility without any effect on amine oxidase activity or its keeping qualities.

The purified amine oxidase preparations used for study of substrate specificity and the kinetics of the oxidation of certain amines were made as follows:

A liver from a rabbit immediately after it was killed (70 to 90 gm.) was ground with ice and water in a blender; then the volume was made up to 500 ml. and the pH adjusted to 8.0 with a glass electrode. After fiber was screened out, the mixture was passed twice through a Sharples super-centrifuge and then placed in Visking tubing and dialyzed for 20 hours into 10 liters of water within a refrigerator. Adjustment of the dialyzed solution to pH 6.0 with acetic acid yielded a flocculent precipitate which was centrifuged out. The precipitate was resuspended in 1 liter of distilled water and the pH adjusted to 6.0, the insoluble solids centrifuged off, and the centrifugate discarded. The solids were then resuspended in water with addition of some concentrated phosphate buffer solution and some 1:1000 phenylmercuric acetate solution and exact adjustment of pH, so that the final solution was 250 ml. in volume and contained 0.1 M sodium phosphate buffer of pH 7.0 and was 1:10,000 in phenylmercuric acetate. This suspension which was used in the enzyme experiments was a pink-red, turbid fluid.

Following a preparation through this procedure with regard to protein



content and enzyme activity showed 9.8 gm. of protein and 440 enzyme units in the original dialysate. The supernatant liquid from the precipitation at pH 6.0 contained 4.7 gm. of protein and about 35 enzyme units, and the wash solution contained 0.7 gm. of protein and about 25 enzyme units. The final enzyme solution contained 3.8 gm. of protein and 280 enzyme units. Thus, the handling losses in this preparation amounted to about 6 per cent of the protein and about 20 per cent of the enzyme activity, but the loss was justified by the gain in activity per unit of protein and other total solid content.

*Combined Influence of pH and Substrate on Activity*—The effect of pH on tyramine oxidation with amine oxidase was studied by Hare (1). The enzyme and substrate were mixed, the pH adjusted to different values, and

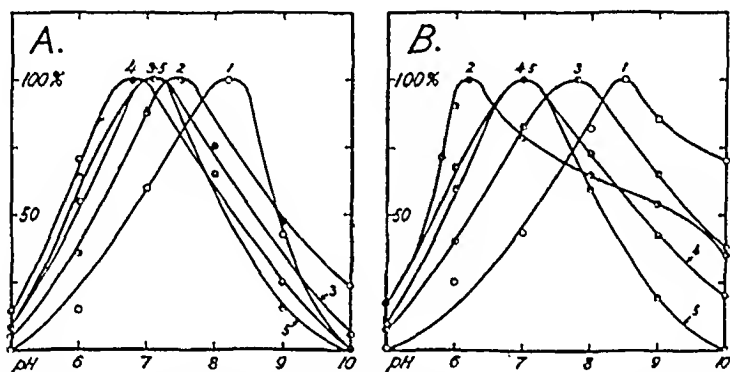


FIG. 2. Relative rates of oxygen uptake against pH for different substrates. The rates are calculated as per cent of rate of each compound at optimum pH. A, Curve 1 butylamine, Curve 2 amylamine, Curve 3 hexylamine, Curve 4 heptylamine, Curve 5 octylamine; B, Curve 1 phenmethylaniline, Curve 2 phenethylaniline, Curve 3 phenpropylaniline, Curve 4 phenbutylaniline, Curve 5 phenamylaniline.

the oxygen uptake of the mixture then determined. The experiments indicated a pH optimum at about 9 to 10, with rapid decrease in activity at both higher and lower pH values; so that oxygen uptake was only about half of the maximum at pH 7. Interpretation of the decrease above pH 10 is complicated in such experiments, for Hare showed that notable destruction of the enzyme activity occurs within 5 minutes at such high alkalinities.

The pH and activity relationship was studied in the present work with a number of different substrates to determine to what extent it would vary within a series of amines. The alkylamines afford a series of compounds that are ionized in water solution to about the same extent, according to available data (see Alles (9)). These compounds only show a variation in  $pK_b$  at 25° from 3.34 to 3.59. As may be noted from Fig. 2, the

optimum pH for these compounds extends from about 8.1 for butylamine through a minimum of about 6.9 for heptylamine, and is around 7.1 to 7.2 for octyl and higher amines.

Results obtained with a series of phenalkylamines ( $\omega$ -phenylalkylamines) are also shown in Fig. 2, B and are of particular interest because good determinations of their ionization constants in water solutions are available from the work of Carothers, Bickford, and Hurwitz (10). Their  $pK_b$  values vary from phenmethylaniline 4.63, phenethylaniline 4.17, phenpropylaniline 3.80, phenbutylaniline 3.60, to phenamylaniline 3.51. The ionization constants of the latter two amines are within the range for

TABLE II

*Maximum Oxidation Rates (Per Cent) Relative to Phenethylaniline*

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Enzyme Preparation .....		A	B	C	Average
Straight chain compounds	Methylaniline.....	0	0	0	0
	Ethylaniline.....	0	0	0	0
	Propylaniline.....	0	0	0	0
	Butylaniline.....	36	62	55	51
	Amylaniline.....	101	102	116	106
	Hexylaniline . . . . .	115	104	123	114
	Heptylaniline.....	129	122	130	127
	Octylaniline. . . . .	133	145	141	140
Branched chain compounds	$\beta$ -Methylpropylaniline (isobutylaniline) . . .	9	8	13	10
	$\beta$ -Methylbutylaniline . . .	71	82	95	83
	$\gamma$ -Methylbutylaniline (isomylaniline)... . .	86	100	113	100
	$\gamma$ -Methylamylaniline. . .	87	92	106	95
	$\delta$ -Methylhexylaniline (isohexylaniline) . . . .	98	100	113	103

alkylamines, indicating the effect of the phenyl group to be lost. The weakest of these bases, phenmethylaniline, requires the most strongly basic environment for maximum activity, but phenethylaniline falls out of any regular series variation in that its maximum activity is at the lowest pH of any of the series. It should be noted that the plot of Fig. 2 is made to indicate most clearly the pH variation for each compound by assigning 100 per cent value to the maximum rate at the pH optimum for each amine separately, and does not indicate that the maximum oxidation rate for the several compounds is the same at their optimum pH. The relative oxidation rates for the same compounds at pH 7.0 alone are given in Tables II and IV.

*Aliphatic Primary Amines As Substrates*—The oxidation system consisted of 2.0 ml. of purified amine oxidase preparation in 0.1 M phosphate of pH 7.0 and 0.3 ml. of water, to which was added at zero time 0.2 ml. of 0.1 M amine salt solution. The amine salts were mostly sulfates, though chlorides were used on occasion without notable anion effect. Readings were taken every 2.5 minutes to determine accurately the form of the  $O_2$  uptake against time relationship, and the rates recorded in the tables are maximum rates, often largely determined from the first few readings. A control of the enzyme preparation without added amine was run at the same time as was a comparison standard containing 0.2 ml. of 0.1 M phenethylamine. Results were discarded if more than a negligible oxygen uptake was noted in the control during the course of the experiment. By repeating such experiments and calculating on the basis of the simultaneous uptake rate observed for phenethylamine average values were obtained as shown in Table II.

The compounds of Table II are all primary carbinamines, and it is to be noted that the branched chain compounds were somewhat less actively oxidized than the straight chain compound of corresponding total number of carbon atoms. The rate of oxidation increases among the series of compounds as the branching of the chain is further removed from the amino group.

A series of secondary carbinamines in which an  $\alpha$ -methyl group was introduced into the series of normal alkylamines was particularly studied. All of the following  $\alpha$ -methylalkylamines were found to be completely unoxidizable under the same conditions as described for the testing of the primary carbinamines:  $\alpha$ -methylethylamine (isopropylamine),  $\alpha$ -methylpropylamine (*sec*-butylamine),  $\alpha$ -methylbutylamine (*sec*-amylamine),  $\alpha$ -methyldimethylamine,  $\alpha$ -methylhexylamine,  $\alpha$ -methylheptylamine,  $\alpha$ -methyloctylamine,  $\alpha$ -methylnonylamine.

A corresponding series of tertiary carbinamines in which two  $\alpha$ -methyl groups were introduced into the series of normal alkylamines was also studied. All of the following  $\alpha,\alpha$ -dimethylalkylamines were completely unoxidizable under the same conditions:  $\alpha,\alpha$ -dimethylethylamine (*tert*-butylamine),  $\alpha,\alpha$ -dimethylpropylamine (*tert*-amylamine),  $\alpha,\alpha$ -dimethylbutylamine,  $\alpha,\alpha$ -dimethyldimethylamine,  $\alpha,\alpha$ -dimethylhexylamine,  $\alpha,\alpha$ -dimethylheptylamine.

Although Bhagvat, Blaschko, and Richter (11) noted no oxidation of cadaverine or putrescine by crude extracts of guinea pig intestine and liver, it appeared desirable to extend the observations to the aliphatic diamines more generally. Those studied were all primary carbinamines and the higher members of the series might be expected to behave more like monoamines, since their groups are too far apart to transmit effects along their

carbon chains. The following compounds were completely unoxidized by amine oxidase under the conditions described for the primary carbin-monamines: ethylenediamine, trimethylenediamine, tetramethylenediamine (putrescine), pentamethylenediamine (cadaverine), hexamethylenediamine, heptamethylenediamine, octamethylenediamine.

One additional point seemed worthy of special investigation with the primary carbinamines as substrates. In comparisons made by Blaschko, Richter, and Schlossmann (4) of the relative effects of crude liver extracts of guinea pig, rat, or pig on various substrates, it is notable that heptylamine is less oxidized in an hour than is isoamylamine or phenethylamine. A like situation was noted in our experiments with guinea pig, cat, and cattle liver extracts, as shown by the data of our Table I in which maximum oxidation rates only are considered. The results with rabbit liver extracts are dissimilar, and it is even more notable that among the series of the aliphatic primary carbinamines heptylamine is the most actively oxidized by rabbit liver extract, while with guinea pig, cat, and cattle liver extracts

TABLE III

*Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine with Purified Cattle Liver Amine Oxidase Preparation*

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Methylamine.....	7	Amylamine... ..	91
Ethylamine.....	8	Hexylamine . . . . .	91
Propylamine.....	75	Heptylamine.. . . .	89
Butylamine.....	117	Octylamine . . . . .	75

one of the lower members of the series represents a maximum for the series. Further investigation of this phenomenon was carried out by purifying a cattle liver extract by following the procedure described for purified preparations of rabbit liver amine oxidase, then carrying out oxidation studies in the same manner as were those described in Table II. The results are shown in Table III. In contrast to the results with amine oxidase preparations from rabbit liver, there is clearly a maximum of activity among this series of amines, and the three lowest members of the series are oxidizable, propylamine being quite notably oxidized, whereas with rabbit liver preparations it is not at all.

*N-Methyl Derivatives of Aliphatic Amines As Substrates*—Although Blaschko, Richter, and Schlossmann (4) found with extracts of guinea pig intestine and liver that the symmetrical dialkylamines and trialkylamines were not oxidized, it seemed important to study a number of alkylmethylamines and alkyl dimethylamines in comparison with the alkyl primary amines. The oxidation system and conditions were kept as for the experi-

ments of Table II; so that the oxidation rates observed with the alkylamines in these experiments were assigned the average values as there given and the oxidation rates of the N-methyl and N-dimethyl derivatives calculated on this basis. The alkyltrimethylammoniums were included in this grouping of compounds, for the only previously investigated compound

TABLE IV

*Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine*

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Butylamine .....	51	Hexylamine.....	114
Butylmethylamine .....	27	Hexylmethylamine.....	46
Butyldimethylamine.....	0	Hexyldimethylamine.....	5
Butyltrimethylammonium..	0	Hexyltrimethylammonium..	0
Amylamine.....	106	Heptylamine.....	127
Amylmethylamine .....	57	Heptylmethylamine.....	70
Amyldimethylamine .....	0	Heptyldimethylamine.....	15
Amyltrimethylammonium ..	0	Heptyltrimethylammonium	0

TABLE V

*Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine*

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

Phenmethylamine (benzylamine)	36	Phenpropylamine ( $\gamma$ -phenylpropylamine)	109
Phenethylamine ( $\beta$ -phenylethylamine)	100	Phenbutylamine ( $\delta$ -phenylbutylamine)	118
$\beta$ -Methylphenethylamine ( $\beta$ -phenylpropylamine)	67	Phenamylamine ( $\epsilon$ -phenylamylamine)	115
Phenethylmethylamine	91	Phenethylethylamine	23
Phenethyldimethylamine	32	Phenethyldiethylamine	0
Phenethyltrimethylammonium	0	Phenethyltriethylammonium	0
Phenethylbenzylamine	0	Di-(phenethylamine)	0
$\beta$ -Hydroxyphenethylamine (phenethanolamine)	22	$\beta$ -Methoxyphenethylamine	0
$\beta$ -Ketophenethylamine ( $\omega$ -aminoacetophenone)	18	$\beta$ -Ethoxyphenethylamine	0

of this general type was hordenine methochloride (11). The results are shown in Table IV.

*Phenylaliphatic Amines As Substrates*—Several compounds of the series of phenylalkyl primary carbinamines have been studied by others under various but unrelatable conditions and found to be capable of being oxidized by liver and some other tissue extracts (1, 4, 7, 11, 12). It was desirable to extend the series of  $\omega$ -phenylalkylamines studied and to include certain derived types, particularly those that have interest because of their con-

siderable physiological activity. The test system was as described for the aliphatic amines and the results (Table V) are therefore placed on a comparable quantitative basis with the data on those compounds as well as with each other.

Series of secondary carbinamines were studied in which an  $\alpha$ -alkyl,  $\alpha$ -phenyl, or  $\alpha$ -phenalkyl group was introduced into the molecule of one of the phenylalkyl primary carbinamines listed in Table V. The conditions of testing were identical with those used for these latter compounds, but in no case was there any oxygen uptake with the following compounds.

$\alpha$ -Methylphenmethylaniline ( $\alpha$ -phenyl-ethylamine)	$\alpha$ -Ethylphenmethylaniline ( $\alpha$ -phenyl-propylamine)
$\alpha$ -Methylphenethylamine (phenisopropylamine)	$\alpha$ -Phenylphenmethylaniline (benzhydramine)
$\alpha$ -Methylphenpropylamine	$\alpha$ -Ethylphenethylamine (benzylethylcarbinamine)
$\alpha$ -Methylphenbutylamine	$\alpha$ -Propylphenethylamine
$\alpha$ -Methylphenamylamine	$\alpha$ -Isopropylphenethylamine
$\alpha,\beta$ -Dimethylphenethylamine ( $\beta$ -methylphenisopropylamine)	$\alpha$ -Benzylphenethylamine (dibenzylcarbinamine)
$\alpha$ -Methylphenethylmethylaniline (phenisopropylmethylaniline)	$\alpha$ -Methylphenethylethylamine (phenisopropylethylamine)
<i>dl,ld</i> - $\beta$ -Hydroxyphenisopropylamine ( <i>DL</i> -norephedrine)	<i>dd,ll</i> - $\beta$ -Hydroxyphenisopropylamine ( <i>DL</i> -norpseudoephedrine)
<i>dl,ld</i> - $\beta$ -Hydroxyphenisopropylmethylaniline ( <i>DL</i> -ephedrine)	<i>dd,ll</i> - $\beta$ -Hydroxyphenisopropylmethylaniline ( <i>DL</i> -pseudoephedrine)

Two tertiary carbinamines were studied in which two  $\alpha$ -methyl groups had been introduced into a couple of the phenylalkyl primary carbinamines previously tested. Testing of these following compounds under the same conditions did not result in any oxygen uptake:  $\alpha,\alpha$ -dimethylphenethylamine,  $\alpha,\alpha$ -dimethyl- $\beta$ -hydroxyphenethylamine.

*Ring-Substituted Phenylaliphatic Amines As Substrates*—The ring-substituted phenethylamines, particularly the phenolic derivatives, have special interest from both biochemical and pharmacological view-points. A few compounds of this type were tested as to their oxidizability by extracts of guinea pig intestine or liver by Blaschko, Richter, and Schlossmann (4). Certain other compounds were later studied (11, 12), but a more systematic study with better quantitative data was needed for any general conclusions as to the effect of any particular type of ring substituent. The effect of the different position of substituents in the ring was also given particular attention. Test conditions were the same as those used for the phenylaliphatic amines. The results are presented in Table VI.

Secondary carbinamines that are  $\alpha$ -methyl derivatives of a number of the compounds listed in Table VI were studied to see whether such type compounds, as in the aliphatic and phenylaliphatic series, withstand the

amine oxidase activity under the standard test conditions employed. The following were studied, and all were completely unoxidized by rabbit liver amine oxidase.

3-Hydroxyphenisopropylamine	3-Methylphenisopropylamine
4-Hydroxyphenisopropylamine (para-drine)	4-Methylphenisopropylamine
3-Methoxyphenisopropylamine	3-Methoxy-4-hydroxyphenisopropylamine
4-Methoxyphenisopropylamine (O-methylpara-drine)	3-Hydroxy-4-methoxyphenisopropylamine
3,4-Dimethoxyphenisopropylamine	3,4-Dihydroxyphenisopropylamine (hydroxyparedrine)
3,4-Methylenedioxyphenisopropylamine	3,4,5-Trimethoxyphenisopropylamine ( $\alpha$ -methylmescaline)
4-Hydroxyphenisopropylmethylamine (N-methylpara-drine, para-drinol)	3,4-Dihydroxyphenisopropylmethylamine

*Enzyme Constants of Amine Oxidase and Amines*—The velocity of the oxidation of the amines with amine oxidase can be expected to follow the well known equation set up by Michaelis and Menten (13) in the form  $v = V_{\max} \cdot (S)/(K_s + (S))$ , where the velocity  $v$  is dependent on the substrate concentration  $S$  and the enzyme-substrate constants  $K_s$  and  $V_{\max}$ .  $K_s$  is the kinetic dissociation constant of the enzyme-substrate compound and  $V_{\max}$  its oxidation rate at infinite substrate concentration. In the tables, such as Tables II, V, and VI, the relative velocities for the different aliphatic and phenylaliphatic amines at a constant concentration may be expressions of differences either in  $K_s$  or  $V_{\max}$ , or both. A number of experiments were made to value these two constants separately for certain aliphatic and phenylaliphatic type compounds to indicate to what extent they may independently vary.

Substrate concentrations were chosen to make the variation in rate of oxygen uptake between the different Warburg vessels as great as possible, and with concentrations to allow giving a uniform spread for the function  $1/S$ . The enzyme concentration used was decreased to half that used in the previous experiments with fixed substrate concentration, so that there was but about 0.5 enzyme unit per 2.5 ml. of total volume in the vessel. The data were plotted to conform to the alternate expression of the rate equation offered by Lineweaver and Burk (14) in the form  $1/v = K_s/V_{\max} \cdot (S) + 1/V_{\max}$ , where a graph of  $1/v$  against  $1/S$  intercepts the  $1/v$  axis at  $1/V_{\max}$ , and has the slope  $K_s/V_{\max}$ . The value of  $1/K_s$  is readily obtained from such a graph as the  $1/S$  value corresponding to half maximum velocity, which is at  $2/V_{\max}$  on the graph, and is indicated in Fig. 3 with light lines.  $v$  represents the oxygen uptake per 15 minutes in Fig. 3.

The scattering of points is assumed to be due to experimental errors,

TABLE VI

*Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine*

At 30° with 0.008 M amine substrates in 0.1 M phosphate, pH 7.0.

2-Hydroxyphenethylamine	43	2-Methylphenethylamine	87
3-Hydroxyphenethylamine	82	3-Methylphenethylamine	68
4-Hydroxyphenethylamine (tyramine)	80	4-Methylphenethylamine	87
2-Methoxyphenethylamine	100	3,4-Dihydroxyphenethylamine (hydroxytyramine)	68
3-Methoxyphenethylamine	98	3-Methoxy-4-hydroxyphenethylamine (homovanillylamine)	22
4-Methoxyphenethylamine (O-methyltyramine)	88	3-Hydroxy-4-methoxyphenethylamine	76
3,4-Dimethoxyphenethylamine	41	3-Methoxy-4,5-methylenedioxyphenethylamine	72
3,4-Methylenedioxyphenethylamine	78	3,4,5-Trimethoxyphenethylamine	0
4-Hydroxyphenethylmethylamine (N-methyltyramine)	70	3,4-Dihydroxyphenethylmethylamine (epinine)	45
4-Hydroxyphenethylmethylamine (hordenine)	25	3,4,5-Trimethoxyphenethylmethylamine (N-methylmescaline)	0
4-Hydroxyphenethanolmethylamine (synephrine)	24	3,4-Dihydroxyphenethanolmethylamine (dl-epinephrine)	10
$\beta$ -Keto-4-hydroxyphenethylmethylamine (synephrine ketone)	8	$\beta$ -Keto-3,4-dihydroxyphenethylmethylamine (adrenalone)	5

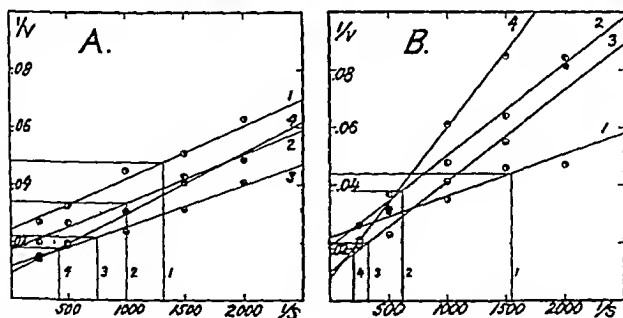


FIG. 3. Reciprocal microliters of oxygen uptake rates against reciprocal molal substrate concentrations at 30° with 2.0 ml. of purified rabbit liver amine oxidase in 0.2 M phosphate buffer, pH 7.0, made up with amine salt solution to a total volume of 2.5 ml. A, Curve 1 amylamine, Curve 2 hexylamine, Curve 3 heptylamine, Curve 4 octylamine; B, Curve 1 phenethylamine, Curve 2 phenpropylamine, Curve 3 phenbutylamine, Curve 4 phenamylamine.



and not to any non-conformity to the rate equations. In Table VII are presented enzyme constants obtained from the data shown in Fig. 3 and similar data for certain other substrates, all determined with a single enzyme preparation.

The variance in these values is indicated by the finding in later experiments, which were carried out with another enzyme preparation made by the same method but from another rabbit liver, of  $1/V_{\max}$  and  $1/K_s$  values for phenethylamine of 0.027 and 1800, and for phenethylmethylamine of 0.025 and 3000. In another experiment, the values for phenethylamine were 0.025 and 1360, and in another with 4-hydroxyphenethylamine, the values were 0.031 and 720.

TABLE VII  
*Constants for Enzyme-Substrate Compounds*

Substrate	$\frac{1}{K_s}$	$\frac{1}{V_{\max}}$	$K_s$	$V_{\max}$
			<i>M</i>	<i>microliters O<sub>2</sub></i> <i>per 15 min.</i>
Amylamine.....	1320	0.021	0.0008	42
Hexylamine.....	1000	0.017	0.0010	59
Heptylamine.....	750	0.011	0.0013	90
Octylamine.....	420	0.009	0.0024	110
Phenethylamine.....	1550	0.022	0.00064	45
Phenpropylamine.....	610	0.019	0.0016	53
Phenbutylamine.....	320	0.010	0.0031	100
Phenamylamine.....	200	0.008	0.0050	120
Phenethylamine.....	1600	0.022	0.00062	45
Phenethylmethylamine.....	3800	0.026	0.00026	38
4-Hydroxyphenethylamine.....	670	0.036	0.0015	28
3,4-Dihydroxyphenethylamine..	350	0.044	0.0029	23

#### DISCUSSION

The considerable species variance that is notable from the data of previous workers and those here presented make it questionable that amine oxidase preparations from various sources may be viewed as but a single enzyme. For the present it seems best to keep in mind particular results as being those found under the set of the given experimental conditions. The way that the maximum of activity varies in a particular homologous series with enzyme from different sources is very marked, and may be taken as good evidence of there being different enzymes present. The relatively marked activity of preparations of guinea pig liver upon phenolic and diphenolic amines also would appear to indicate a special character in such respect for the species. That such special characters are a function

of the enzyme itself, rather than due to associated impurities, is indicated by the retention of the relative specificities of rabbit and cattle liver extracts as they are subjected to purification procedures.

While the data given in Tables II through VI show what the relationships of substrate to activity are for the particular set of experimental conditions, the extent to which such data can be taken to indicate expected relationships under other conditions is limited. As shown by the data of Fig. 2, even in a series of compounds having constant dissociation constants, marked variations in activity with pH are seen, and relative activities among a series of compounds are dependent upon the pH of interest.

With regard to relationships between structure and oxidizability by amine oxidase, the differences between primary, secondary, or tertiary carbinamines were most clearly brought out and are of greatest interest because of the importance of this difference in structure for chemical reactivity of the C—N bond. The more expected differences between primary, secondary, and tertiary amines were found to be quite highly dependent upon the structure of the rest of the molecule, and difficult to cover with comprehensive rules. The effect of various substituents in the benzene ring or the side chain of phenethylamines is marked in most cases. The decreased oxidizability of hydroxy and N-methyl compounds is of most interest in connection with their physiological activities.

It should be noted from the data of Table VII that in both aliphatic and phenylaliphatic amines  $K_s$  and  $V_{max}$  both increase with chain length of the radical attached to the amino group. The trend of variation of both constants with increasing number of carbon atoms in a homologous series tends to keep the observed rates of oxidation in the series relatively constant when compared at a given substrate concentration. If the rates were determined at relatively high substrate concentrations (corresponding to velocities  $4/5$  of  $V_{max}$  of the highest  $V_{max}$  of the series), the variation in velocity would be primarily dependent on the variation in  $V_{max}$  and independent of  $K_s$ . Under such conditions an increase in the velocity of oxidation would be observed through the series of substrates rather than the passing through a maximum effect in the series as is seen in the experiments of Tables II and V.

The relative velocity of oxidation of these various substrates by amine oxidase by preparations from a liver of a single species is therefore not only dependent on the pH of the determinations, but also on the particular concentration of substrate at which the determination is made. Together, these various factors make it impossible to use oxidation velocities of a series of amines under particular conditions as an exact basis for prediction of relative rates under other conditions of pH and concentration.

Some calculations may be made with respect to the likelihood that amine

oxidase plays a physiological rôle in the inactivation of certain types of amines in the body. By way of example, consider the oxidation rate of tyramine in a concentration of  $10^{-5}$  M with an enzyme concentration of about 0.2 enzyme unit per ml. Substitution in the rate equation  $v = V_{\max} (S)/(K_s + (S))$  of  $10^{-5}$  for  $S$  and the values of  $V_{\max}$ , and  $K_s$  for tyramine given in Table VII gives  $v$  about 0.2 microliter of  $O_2$  per minute, or about  $10^{-8}$  mole of  $O_2$  per minute. On the basis that physiological inactivation would occur with an uptake of 1 mole of  $O_2$  per mole,  $10^{-6}$  mole of tyramine would require about 100 minutes to be inactivated with an enzyme concentration of 0.2 enzyme unit per ml. Such conditions and amounts of tyramine would be approximated in the blood stream of an experimental animal immediately following the intravenous injection of  $10^{-6}$  mole per kilo (0.18 mg. per kilo of hydrochloride) of tyramine. The studies of Clark and Raventos (15) on the relationship between dosage and duration of physiological action of tyramine in cats and man showed that a dosage of  $10^{-6}$  mole per kilo is inactivated in 20 minutes. The liver of the cat contains somewhat more enzyme than that of the rabbit, as shown in Table I, and the over-all concentration in rabbit liver is about 5 enzyme units per gm. Under conditions *in vivo*, the temperature is higher and the pH is somewhat more optimum for the enzyme-tyramine oxidation, according to Hare (1). There is consequently a fair agreement between data *in vitro* and *in vivo*, indicating that indeed the biochemical enzyme studies of amine oxidase may be expected to add much to the physiological perfusion studies of Ewins and Laidlaw (16), and of Guggenheim and Löf- fler (17) on the destruction of this type of compound in the body.

In passing, it should be pointed out that, although we have not been able to determine the enzyme constants with epinephrine and must leave our studies, it is probable that there will not be a close correspondence of data *in vitro* and *in vivo* with this compound. Our observations do show that  $V_{\max}/K_s$  is much lower for this compound than for tyramine, while the *in vivo* data of Clark and Raventos (15) show it to be much more rapidly oxidized than tyramine in the body. Such considerations are in accord with the studies of Richter and Tingey (18) and are more directly confirmed by the establishment of another detoxification pathway for diphenolic amines by Richter (19) and later by Richter and MacIntosh (20).

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# INHIBITOR SPECIFICITY OF AMINE OXIDASE

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In their studies of the oxidation of adrenalin and other amines by amine oxidase preparations, Blaschko, Richter, and Schlossmann (1) found that *l*-ephedrine, triethylamine, triisoomylamine, and *dl*-1-hydroxy-2-hydrindeneamine are not only not oxidized, but inhibit the action of the enzyme upon isoamylamine. The conclusion was drawn that this inhibition was of the competitive type, because some data were available which indicated a dependence of degree of inhibition upon the concentration of both enzyme and substrate. Natural *l*-ephedrine and the related compounds, suprifren and veritol, were later reported by Blaschko (2) to inhibit amine oxidase in its action upon *l*-sympathol. Some further extension of such studies to include benzedrine, and the finding that this compound and veritol are stronger inhibitors of amine oxidase than are ephedrine isomers in the particular system studied, led Blaschko (3) to conclude there was agreement between the inhibiting effects *in vitro* on amine oxidase and their awakening properties in animals which are pharmacological effects upon the central nervous system.

Previous to this, Gaddum and Kwiatkowski (4) had advanced the theory that ephedrine action in animals was due to its inhibition of adrenalin oxidation by amine oxidase in the body. This was based on demonstration that in the perfused rabbit ear, sympathetic stimulation liberated a substance indistinguishable from adrenalin, and that this substance like adrenalin is increased in certain of its physiological activities by addition of ephedrine. The study by Richter and Tingey (5) of the kinetics of the rate of inactivation of adrenalin by amine oxidase and the degree of inhibition of this oxidation by ephedrine permitted calculations which did not support the view that amine oxidase is specifically concerned in the inactivation of adrenalin in the body. Further evidence obtained by Richter (6) and Richter and MacIntosh (7) from their isolation of some of the metabolic products excreted in the urine after administration of adrenalin and other *o*-diphenolic pressor amines makes it quite certain that adrenalin is not considerably acted on by amine oxidase in passing through the body.

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Thus, although at the present time it appears clear that amine oxidase oxidation of adrenalin, or other *o*-diphenolic pressor amines such as were studied by Richter (6), does not play a significant physiological rôle, it is equally clear that the inactivation of aliphatic amines, phenethylamine and probably 4-hydroxyphenethylamine (tyramine), does predominantly take place by amine oxidase oxidation. In view of the evidence from the experiments of Ewins and Laidlaw (8) and a later study by Guggenheim and Löffler (9), such amine oxidations chiefly occur in the liver. In the present studies, an attempt was made to value quantitatively the inhibition of some of these particular type compounds by certain types of amines which are not themselves oxidized by the enzyme system (see Alles and Heegaard (10)).

#### EXPERIMENTAL

Purified amine oxidase preparations from rabbit liver were used throughout the present work. They were prepared as described by Alles and Heegaard (10) and experiments in which there was any considerable uptake of oxygen by the control alone were discarded along with that particular enzyme preparation. The hydrogen ion activity was maintained uniformly at pH 7.0 in all experiments, though it is expected that there can be observed a relation between pH and degree of inhibition. Such relation will probably have a considerable variation even among closely related series of compounds, just as the variation in amine oxidase activity with pH depends on the particular substrate acted upon, as shown by our studies (10).

It was most desirable to obtain data indicating inhibitor effects with a considerable number of compounds of different types, and then to make a critical study with a particular inhibitor and various substrate types to determine whether such inhibition was competitive.

The term observed activity in any experiment refers to the maximum rate of oxygen uptake. This often occurs only during the first few observations of any experiment, particularly with aliphatic amine substrates, and is determined as the tangent to the initial part of the plotted curve of observed oxygen uptakes. Such valuation of rate minimizes the effects of uncontrolled inhibiting variables that enter into rate valuations dependent upon a single observation made after some considerable time.

*Aliphatic Amines As Inhibitors of Aliphatic Amine Substrates*—Experiments were made to value the relative inhibiting effects of a series of secondary carbinamines. The oxidation system consisted of 2.0 ml. of purified amine oxidase preparation in 0.1 M phosphate buffer of pH 7.0, to which were added 0.1 ml. of 0.1 M amylamine as a neutral solution of its sulfate and 0.4 ml. of variable concentrations of inhibitor amine sulfate or chloride in neutral solution. The final concentration of amylamine in the mixture

was 0.004 M, and the concentrations of inhibiting amines were 0.004, 0.008, 0.012, and 0.016 M, giving the inhibitor- $\alpha$ -methylamine ratios presented in Table I. The oxygen uptake at 30° was followed with the Warburg apparatus and calculated to per cent of the uptake of the control sample with substrate but without any inhibitor.

The inhibiting effect in this series of data was clearly at a maximum with  $\alpha$ -methylmethylamine. In further experiments, in which the ratio of  $\alpha$ -methylmethylamine was 4:1, no inhibition was observed.  $\alpha$ -Methyloctylamine and  $\alpha$ -methylnonylamine were found to be less active inhibitors than  $\alpha$ -methyl-

TABLE I  
*Inhibitor-Amylamine Ratios*

In per cent of O<sub>2</sub> uptake, based on the rate without inhibitor.

<u>Inhibitor</u> <u>Amylamine</u>	0	1:1	2:1	3:1	4:1
$\alpha$ -Methylpropylamine ( <i>sec</i> -butylamine).....	100	101	98	92	88
$\alpha$ -Methylbutylamine ( <i>sec</i> -amylamine).....	100	86	74	64	55
$\alpha$ -Methylmethylamine.....	100	73	62	38	36
$\alpha$ -Methylhexylamine.....	100	90	73	53	42
$\alpha$ -Methylheptylamine.....	100	93	79	60	49

TABLE II  
*Effect of  $\alpha$ -Methylbutylamine As Inhibitor*

In per cent of O<sub>2</sub> uptake, based on the rate without inhibitor.

<u><math>\alpha</math>-Methylbutylamine</u> <u>Substrate</u>	0	1:1	2:1	3:1	4:1
Butylamine.....	100	56	38	34	25
Amylamine.....	100	86	74	64	55
Hexylamine.....	100	91	84	83	79
Heptylamine.....	100	96	93	92	70

heptylamine. To cover a variance in substrate,  $\alpha$ -methylbutylamine was studied as an inhibitor of several primary carbinamine substrates of 0.004 M final concentration in the oxidation system. The results are shown in Table II.

A series of tertiary carbinamines was studied under similar conditions as inhibitors of the primary carbinamine, butylamine. There appeared to be an increased inhibitor effect with larger molecular size. The results obtained with 4:1 inhibitor-substrate were as follows:  $\alpha$ ,  $\alpha$ -dimethylethylamine (*tert*-butylamine) 100,  $\alpha$ ,  $\alpha$ -dimethylpropylamine (*tert*-amylamine) 91,



$\alpha$ ,  $\alpha$ -dimethylbutylamine 84,  $\alpha$ ,  $\alpha$ -dimethylamylamine 61,  $\alpha$ ,  $\alpha$ -dimethylhexylamine 45.

Although Blaschko (11) noted, under his conditions of study, that eadaverine did not act as an inhibitor to the enzymic oxidation of amylamine or synephrine, observations were extended to include other aliphatic diamines. With 0.004 M amylamine as substrate and a 4:1 ratio of the  $\omega$ -alkylenediamines, there was no exhibition of any notable inhibitor effect with tetramethylene-, pentamethylene-, hexamethylene-, heptamethylene-, and octamethylenediamine.

Another type of compound was of interest because of its relatively complete ionization in water solution. The alkyltrimethylammoniums were found not to be oxidized by amine oxidase in our earlier work (10). With 0.004 M amylamine as substrate and a 4:1 ratio of butyl-, amyl-, hexyl-,

TABLE III

*Effect of  $\alpha$ -Methylphenalkylamines As Inhibitor*

In per cent of O<sub>2</sub> uptake, based on the rate without inhibitor.

<u>Inhibitor</u> Amylamine	0	2:1	4:1
$\alpha$ -Methylphenmethylamine ( $\alpha$ -phenylethylamine).....	100	72	26
$\alpha$ -Methylphenethylamine (phenisopropylamine).....	100	80	44
$\alpha$ -Methylphenpropylamine.....	100	70	20
$\alpha$ -Methylphenbutylamine.....	100	87	27
$\alpha$ -Methylphenamylamine.....	100	90	35

or heptyltrimethylammonium, no considerable inhibitor effect was observed.

*Phenylaliphatic Amines As Inhibitors of Amine Substrates*—While Blaschko (3) found that benzedrine (phenisopropylamine) and certain of its derivatives acted as inhibitors of amine oxidase under particular experimental conditions, his findings offered only meager experimental evidence for the generalization that inhibitor effect is a property of secondary carbamines. A series of  $\alpha$ -methylphenalkylamines was studied by us for their inhibitory effect on the oxidation of amylamine. The concentration of amylamine was 0.004 M, and other conditions were the same as in the studies of aliphatic amines as inhibitors (see Table III).

The inhibiting effect of certain types of derivatives of  $\alpha$ -methylphenethylamine is of considerable interest because of possible relation to their physiological actions. A number of such derivatives (Table IV) were tested with amylamine as the substrate at 0.004 M and an inhibitor-amylamine ratio of 4:1.

Owing to a lack of time to complete more extensive studies, the observations of inhibition by secondary phenylaliphatic amines were extended to different amine substrates only with  $\alpha$ -methylphenethylamine itself. Our previous determinations (10) of the kinetic dissociation constants of the enzyme-substrate complex of rabbit liver amine oxidase with aliphatic and phenylaliphatic amines indicated that these two types of compounds did not differ notably in their enzyme-substrate constants. Consequently, it was expected that any inhibitor would exhibit about the same degree of inhibition on these two types of substrates. Dissociation constants  $K_i$  for enzyme-tyramine and enzyme-hydroxytyramine had been found to be

TABLE IV

*Effect of Methylphenethylamines As Inhibitor*

In per cent of  $O_2$  uptake, based on the rate without inhibitor.

<u>Inhibitor</u> Amylamine	4:1	<u>Inhibitor</u> Amylamine	4:1
$\alpha$ -Methylphenethylmethylamine	48	$\alpha$ -Methyl-3-hydroxyphenethyl-amine	65
$\alpha$ -Methylphenethylethylamine	39	$\alpha$ -Methyl-4-hydroxyphenethyl-amine	75
$\alpha$ -Methylphenethyldimethyl-amine	41	$\alpha$ -Methyl-4-hydroxyphenethyl-methylamine	95
$\alpha,\beta$ -Dimethylphenethylamine	71	$\alpha$ -Methyl-4-methylphenethyl-amine	60
$\alpha$ -Methyl- $\beta$ -hydroxyphenethyl-amine ( <i>DL</i> -norephedrine)	97	$\alpha$ -Methyl-4-methoxyphenethyl-amine	64
$\alpha$ -Methyl- $\beta$ -hydroxyphenethyl-amine ( <i>DL</i> -norpseudoephedrine)	99	$\alpha$ -Methyl-3,4-dihydroxyphenethylamine	92
$\alpha$ -Methyl- $\beta$ -hydroxyphenethyl-methylamine ( <i>DL</i> -ephedrine)	79	$\alpha$ -Methyl-3,4-dimethoxyphenethylamine	67
$\alpha$ -Methyl- $\beta$ -hydroxyphenethyl-methylamine ( <i>DL</i> -pseudoephedrine)	85	$\alpha$ -Methyl-3,4-methylenedioxy-phenethylamine	60
$\alpha,\alpha$ -Dimethylphenethylamine	42	$\alpha$ -Methyl-3,4,5-trimethoxy-phenethylamine	80
$\alpha,\alpha$ -Dimethyl- $\beta$ -hydroxyphenethylamine	79		

notably greater, and it was expected that these two amines would be more readily inhibited than either an aliphatic or phenylaliphatic amine. Simultaneous observations of the four types of amines in 0.004 M substrate concentrations alone and in the presence of  $\alpha$ -methylphenethylamine (phenisopropylamine) gave the results shown in Table V.

The particular differences found in the effect with phenisopropylamine added to these different substrates were surprising, and some further studies were made to determine whether indeed competitive inhibition could be observed with phenisopropylamine and these different substrates.

*Phenisopropylamine Inhibition of Amine Oxidase and Amines—Enzyme*

properties are determined chiefly by means of kinetic studies. Competitive inhibition may be distinguished from other types primarily by conforming in its kinetics to the rate equations derivable from the equilibrium  $2E + S + I \rightleftharpoons ES \text{ (active)} + EI \text{ (inactive)}$  where  $E$  represents the enzyme,  $S$  the substrate, and  $I$  the inhibitor. In this simplest case, the rate relations can be represented in the reciprocal form by

$$\frac{1}{v} = \frac{1}{V_{\max.}} \left( K_s + \frac{K_s}{K_i} I \right) \left( \frac{1}{S} \right) + \frac{1}{V_{\max.}}$$

as given by Lineweaver and Burk (12). Here  $v$  is the reaction rate,  $V_{\max.}$  the rate at infinitely high substrate concentration,  $S$  and  $I$  the molal concentrations of substrate and inhibitor, while  $K_s$  and  $K_i$  are the kinetic dissociation constants of the enzyme-substrate and enzyme-inhibitor compounds.

TABLE V

*Effect of Phenisopropylamine As Inhibitor*

In per cent of  $O_2$  uptake, based on the rate without inhibitor.

<u>Phenisopropylamine</u> Substrate	0	4:1	16:1
Amylamine.....	100	45	19
Phenethylamine.....	100	113	144
4-Hydroxyphenethylamine (tyramine).....	100	26	11
3,4-Dihydroxyphenethylamine (hydroxy-tyramine).....	100	20	13

Data were obtained to value the constants by choosing substrate concentrations as in the kinetic studies of our previous work with these same substrates (10). The enzyme concentration in the experiments was again about 0.5 enzyme unit per 2.5 ml. of total volume. Considerable difficulty was encountered in finding the suitable, but necessarily different, concentrations of phenisopropylamine for working with each substrate to get data of sufficient precision. These data were plotted as  $1/v$  against  $1/S$  so that the  $1/v$  axis intercept is  $1/V_{\max.}$  and the  $1/S$  value of  $2/V_{\max.}$  is  $1/K_s$  or  $1/(K_s + (K_s/K_i)I)$  in the presence of the inhibitor. From these values, which can be read directly from the graphs of Fig. 1, are calculated the values of  $V_{\max.}$ ,  $K_s$ , and  $K_i$ .

It is immediately apparent from Fig. 1 that phenisopropylamine is many times more active an inhibitor with phenethylamine as the substrate than with amylamine. Similar experiments were carried at with tyramine and hydroxytyramine as the substrates, and the valuations of the enzyme constants are given in Table VI.

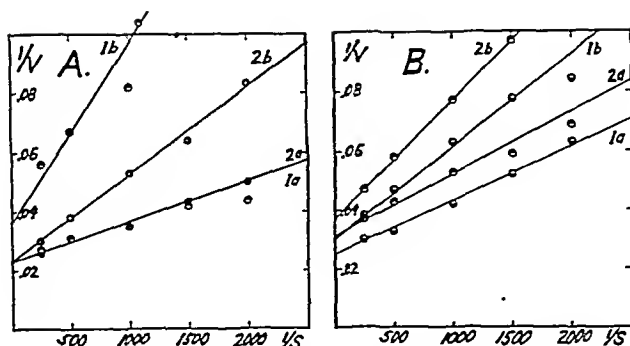


FIG. 1. Reciprocal microliters per 15 minute oxygen uptake rates against reciprocal molal substrate concentrations. At 30° with 2.0 ml. of purified rabbit liver amine oxidase in 0.2 M phosphate buffer, pH 7.0, made up with amine salt solution alone or with inhibitor amine salt to a total volume of 2.5 ml. A, Curves 1a and 2a, amylamine without added inhibitor; Curve 1b, amylamine and 0.0020 M phenisopropylamine; Curve 2b, amylamine and 0.0010 M phenisopropylamine; B, Curves 1a and 2a, phenethylamine without added inhibitor; Curves 1b and 2b, phenethylamine and 0.020 M phenisopropylamine.

TABLE VI  
Enzyme-Substrate and Enzyme-Inhibitor Constants

Substrate used in variable concentration	Phenisopropylamine concentration	$\frac{1}{V_{\max.}}$	$\frac{1}{K_s}$	$\frac{1}{K_s + \frac{K_s}{K_i} I}$	$V_{\max.}$	$K_s$	$K_i$
	M				micro-liters O <sub>2</sub> per min.	M	M
Amylamine	0.0000	0.023	1650		42	0.00061	
"	0.0020	0.036		600	28		0.0011
"	0.0000	0.023	1650		42	0.00061	
"	0.0010	0.023		780	42		0.0009
Phenethylamine	0.0000	0.025	1360		40	0.00074	
"	0.0200	0.030		950	33		0.047
"	0.0000	0.031	1450		32	0.00069	
"	0.0200	0.037		920	27		0.035
4-Hydroxyphenethylamine	0.0000	0.031	720		32	0.0014	
"	0.0040	0.037		380	27		0.0045
3,4-Dihydroxyphenethylamine	0.0000	0.042	510		24	0.0020	
"	0.0040	0.054		230	19		0.0033

Notable in the data of Table VI is the fair agreement between  $1/V_{\max.}$  values for a given substrate with and without inhibitor, which affords the

simplest test of the competitive nature of the inhibitory process. Some small changes do occur, but they approximate the variations that occur on occasion between different experiments. While the experiments with the amylamine and hydroxytyramine were made with but one enzyme preparation, the experiments with phenethylamine were made with two different preparations, and those with tyramine with another. The difference in  $V_{max}$ , between the two experiments with tyramine without inhibitor simply represents the variance in activity of the enzyme preparations.

Even more notable in these experiments are the marked variations in  $K_i$ , dependent upon the substrate competed with. While phenisopropylamine is one-third to one-fourth as active an inhibitor for tyramine and hydroxytyramine as for amylamine, it is only one-thirtieth to one-fortieth as active for phenethylamine inhibition.

#### DISCUSSION

While the data given with regard to the inhibition of amine oxidase oxidation of amylamine as a substrate in fixed concentration do show a large number of compounds to act as inhibitors, the extent to which such data can be taken to indicate expected relationships under other conditions is limited. This is probably particularly so if other substrates are to be considered by analogy to amylamine.

With regard to relationships between structure and inhibition of amine oxidase upon a single substrate, the secondary and tertiary carbinamines are very generally inhibitors. The molecular size of the alkyl and phenyl-alkyl secondary carbinamines would seem to have some specificity with regard to the inhibitor effect, with  $\alpha$ -methylamylamine and  $\alpha$ -methyl-phenylpropylamine appearing to be slightly the most active in their respective series. The generally decreased inhibitor activity of hydroxy derivatives in the side chain or ring of phenethylamines is of most interest in connection with their physiological activities.

The great differences in the inhibitor effect of phenisopropylamine when kinetically valued against particular type substrates show that, while the inhibition exhibited may be an equilibrium situation, it is inadequately considered as an equilibrium only involving  $2E + S + I \rightleftharpoons ES + EI$ , or it may be that the limiting rate of the reaction is not only the oxidation rate of  $ES$ , but also that rates of other reactions are slower than, or comparable to, such oxidation rate. With regard to the equilibrium components, it must be remembered that there are some qualitative rate observations with extracts of liver and other organs of various species that seem to be most easily explained by assuming the presence of several types of amine oxidase enzymes.

The set-up of rate equations for the combination of  $E + S \rightarrow ES$ , together with the dissociation of  $ES \rightarrow E + S$  and the conversion of  $ES \rightarrow E + R$  (the oxidized product) for the analysis of all possible rates controlling the kinetics of reaction between enzyme and substrate, has been carried out by Haldane (13). Such analysis could be extended in the present case to include  $E + I \rightarrow EI$  and  $EI \rightarrow E + I$  as independent rates. By extending rate observations in a systematic way with variance of enzyme, substrate, and inhibitor concentrations independently, such an analysis might be permitted to be made, but we must leave this problem for others.

The increase in oxidation rate observed when 4:1 and 16:1 ratios of phenisopropylamine were used with 0.004 M phenethylamine as substrate appears to be a real effect. It has been noted with certain other secondary carbinamines under some substrate concentrations and particular secondary carbinamine-substrate ratios. The phenomenon has not been investigated, but may be related to the observations of Mann and Quastel (14) who found in brain oxidations with particular substrates that the binding of produced aldehydes by phenisopropylamine caused an over-all increase in oxidation rate. It may be that this effect most notably enters into the lowered inhibitor activity of phenisopropylamine upon phenethylamine as the substrate.

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# ANTIOXIDANT ACTIVITY IN SOURCES OF THE B VITAMINS

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The oxidative destruction of the carcinogen, N,N-dimethylaminoazobenzene, commonly called butter yellow, has been observed in a synthetic ration containing linoleic acid (1). This destruction, which takes place presumably through the action of peroxides or some intermediate oxides of the unsaturated fatty acid, may be prevented by the addition of brown (unpolished) rice to the diet mixture. White (polished) rice exhibits antioxygenic activity to a lesser degree than does brown rice, a fact which suggests that the antioxidant is concentrated in the rice bran. Demonstration of the presence of an antioxidant in an aqueous extract of rice bran, a commercial preparation of the B complex, prompted the present study concerning other sources of the B vitamins, in addition to each of the B vitamins for antioxygenic activity.

## EXPERIMENTAL

The decolorization of butter yellow by linoleic acid in the presence of air is a surface phenomenon; it does not take place in the homogeneous solution of the dye in oil (1). For this reason the bleaching reactions were studied in rations consisting of 8.4 gm. of corn-starch and 1.6 gm. of butter yellow-linoleic acid<sup>1</sup> mixture. Butter yellow was present in the mixture in the amount of 0.6 mg. per gm. of the ration. The material to be tested for antioxidant activity was mixed into the ration either as a solution or in the dry state. The oxidation was permitted to take place at room temperature for 21 days. At various intervals 1.5 gm. of the ration were withdrawn and extracted with 25 cc. of chloroform. The decrease in color of the chloroform extract, determined with the Klett-Summerson photoelectric colorimeter (blue filter, No. 42), gave a measure of the destruction of the butter yellow. The accompanying oxidation of the fatty acid was followed by the decrease in iodine number, as determined by the method of Hanus (2). The results obtained with the above method were checked qualitatively with an accelerated oxidation at 50° for 16 hours.

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<sup>1</sup> "Linoleic acid, refined light," from the Glyco Products Company, Inc., Brooklyn, New York. The iodine number of the different lots varied from 126 to 140.



### Results

*Comparison of Free Linoleic Acid with Its Esters in Destruction of Butter Yellow*—In an earlier study involving the production of malignant hepatoma in rats by feeding butter yellow (1) it was observed that the bright yellow color of the diet containing linoleic acid and butter yellow faded almost completely in 4 days even at room temperature. In contrast to this, a diet which contained an equivalent amount of unsaturated fat in the form of lard retained its color for over a period of several months. These findings seem to indicate that the free acid has greater destructive effect on the dye than have its esters. This assumption was confirmed by using purified samples of linoleic acid and methyl linolate<sup>2</sup> in the diet mixtures. In a 21 day experiment at room temperature the iodine number of the linoleic acid (originally 180) had dropped to 67.4 by the 5th day with an 85 per cent destruction of the butter yellow. In the case of the methyl linolate ( $I_2$  No. 171.9) the iodine number even at the 8th day was 150.0 and there was no detectable destruction of butter yellow. However, at the end of 21 days, almost complete destruction of the dye and oxidation of the fat had occurred in both diets. In a diet containing an equal amount of lard there was no change in iodine number or butter yellow concentration after 21 days.

*Stabilizing Effect of Cereal Grains*—Cereal flours, particularly oat flour, have been suggested as antioxidants for foods which are subject to oxidative deterioration (3). The finding that the linoleic acid-butter yellow system is stabilized in the presence of brown or white rice led to the comparison of the antioxygenic activity of other cereal grains in this same system. Oats, corn, and wheat (ground whole grain being used in each case) were substituted for corn-starch in the experimental mixture. At the end of 21 days, all three mixtures showed little change in the concentration of the butter yellow; namely, 0.54, 0.57, and 0.52 mg. per gm. of diet in the oat, corn, and wheat diet respectively. The iodine number, determined on the wheat mixture extract, was 140.8, thus indicating no oxidation of the fatty acid. Avenex, a commercial antioxidant prepared from oat flour, also exerted complete protection when tested at a 10 per cent level. At a 5 percent level the protection was somewhat less, the concentration of the butter yellow at the 21st day being 0.31 mg. per gm. of diet and the iodine number 100.9.

*Heat Lability of Antioxidants in Cereal Grains*—In all experiments reported in this paper on the effect of heat on antioxidant activity, the treatment involved moist heat under pressure; *i.e.*, the test material was thoroughly mixed with water and the resulting suspension autoclaved for  $\frac{1}{2}$  hour

<sup>2</sup> Purified samples of linoleic acid and methyl linolate were kindly furnished by J. B. Brown of the Department of Physiological Chemistry, The Ohio State University, Columbus.

at 120°. Unless otherwise stated, the pH of the suspension was neutral. The data presented in Table I clearly demonstrate the heat inactivation of the antioxidants in brown rice, white rice, and Avenex.

*Antioxidant Activity of Rice Bran Extract, Heat Treatment, and Dialysis*—The water-soluble nature of the antioxidant of brown rice became evident from experiments in which a crude aqueous extract of rice bran<sup>3</sup> was used. The great activity of such an extract as well as its reaction to heat treatment is shown in Table II. With the admixture of aqueous rice bran ex-

TABLE I  
*Heat Lability of Antioxidants in Cereal Grains*

Experiment No.	Diet mixture	5th day		8th day		21st day	
		Butter yellow per gm. diet	Iodine No.	Butter yellow per gm. diet	Iodine No.	Butter yellow per gm. diet	Iodine No.
		mg.		mg.		mg.	
1	(1) 8.4 gm. brown rice + 1.6 gm. L-BY*	0.69	134.0	0.66	133.7	0.59	134.7
	(2) 8.4 gm. brown rice, autoclaved + 1.6 gm. L-BY	0.63	125.9	0.59	125.7	0.11	75.6
	(3) 8.4 gm. white rice + 1.6 gm. L-BY	0.66	123.8	0.49	125.2	0.09	64.7
	(4) 8.4 gm. white rice, autoclaved + 1.6 gm. L-BY	0.20	82.8	0.11	57.0		
2	(1) 8.4 gm. corn-starch + 1.6 gm. L-BY	0.28		0.10		0.07	49.2
	(2) (1) with 1 gm. Avenex	0.51		0.44		0.55	125.0
	(3) (1) " 0.5 gm. Avenex	0.49		0.43		0.31	100.9
	(4) (1) " 0.5 " " autoclaved	0.29		0.11		0.08	50.9
3	(1) 1.0 gm. Avenex	0.49		0.34		0.31	134.5†
	(2) Control	0.27		0.07		0.07	

\* Butter yellow dissolved in linoleic acid so that the final concentration is 0.6 mg. per gm. of diet.

† After 4 months, the butter yellow concentration was 0.24 mg. per gm. of diet; the iodine number 129.2.

tract, linoleic acid and, in further consequence, butter yellow could be protected from oxidative decomposition for at least 4 to 5 months, whereas, in the absence of any supplementary antioxidant, autoxidation became very noticeable in the short time of 5 to 8 days. Under the experimental conditions chosen amounts of 0.07 cc. of rice bran extract and even 0.03 cc. have shown definite protection up to the 21st day of the experiment.

When the extract was autoclaved, the pH proved to be an important

\* Kindly obtained from the S. M. A. Corporation, Chagrin Falls, Ohio.

factor. With neutral or acid pH there was a loss in antioxidant activity, while autoclaving at pH 10 appeared to exert a definitely protective effect.

The fact that aqueous rice bran extract is capable of delaying the autoxidation of linoleic acid suggested that the antioxidant present in this aqueous extract might be at least partly soluble in linoleic acid. Upon this supposition, a suspension of brown rice in linoleic acid was heated for 15 minutes at 60°. After centrifuging, the stability of the linoleic acid was measured

TABLE II  
*Antioxidant Activity of Rice Bran Extract*

Experiment No.	Rice bran extract added to basic diet*	Butter yellow per gm. diet			Iodine No. (21st day)
		5th day	8th day	21st day	
	cc.	mg.	mg.	mg.	
1	0.67	0.59	0.53	0.57†	
	Control (no supplement)	0.35	0.09	0.06	
2	0.33	0.68	0.71	0.57	131.5‡
	0.33 (Autoclaved)		0.49	0.39§	
	Control	0.27	0.12		
3	0.07	0.60	0.66	0.52	
	0.07 (Autoclaved)	0.66	0.66	0.43	130.4
	0.03	0.60	0.60	0.29	126.8
	0.01	0.36	0.27	0.07	51.0
	Control	0.31	0.17	0.07	
4	0.03	0.36	0.26	0.09	
	0.03 (Autoclaved, pH 3)	0.36	0.28	0.08	
	0.03 ( " " 10)	0.43	0.33	0.40	
5	0.03	0.27		0.11	53.2
	0.03 (Autoclaved, pH 10)	0.40		0.15	86.3
	Control	0.14		0.08	49.0

\* 8.4 gm. of corn-starch + 1.6 gm. of linoleic acid-butter yellow.

† After 5 months, the butter yellow concentration was 0.48 mg. per gm. of diet.

‡ After 4 months, the butter yellow concentration was 0.40 mg. per gm. of diet; the iodine number 121.5.

§ After 4 months, the butter yellow concentration was 0.06 mg. per gm. of diet; the iodine number 43.1.

in the usual manner. This treatment afforded protection from autoxidation for 8 days; at this time the concentration of the butter yellow was 0.53 mg. per gm. of diet. From then on the oxidation proceeded rapidly and by the 21st day the butter yellow concentration was 0.07 mg. per gm. and the iodine number of the fatty acid 47.8. This finding is in good agreement with the previous report (4) on the slight fat solubility of the antioxidants occurring in grains.

Dialysis of rice bran extract in a cellophane bag against frequent changes

of distilled water resulted in a complete loss of antioxygenic activity in the dialyzed solution. A quantitative recovery of antioxidant activity from the dialysate was accomplished.

*Antioxidant Activity in Other Sources of Vitamin B Complex*—The remarkably high antioxidant activity of aqueous rice bran extract which is known to be a rich source of the vitamin B complex suggested the study of other sources of the vitamin B complex with regard to the presence of antioxidants. The results of these investigations are summarized in Tables III and IV. The data obtained make evident the antioxidant property of

TABLE III  
*Antioxidant Activity of Brewers' Yeast*

Experiment No.	Supplement to basic diet	Buttery yellow per gm. diet			Iodine No. (21st day)
		5th day	8th day	21st day	
		mg.	mg.	mg.	
1	0.5 gm. brewers' yeast	0.43	0.52	0.43	129.3*
	0.5 " " " autoclaved	0.41	0.37	0.06	56.0
	0.5 " Squibb's Natuplex B	0.50	0.41	0.39	126.8†
	1.0 cc. 30% Natuplex B	0.43	0.50	0.38	128.8‡
	1.0 " 30% " " autoclaved	0.43	0.46	0.40	129.1§
2	0.5 gm. brewers' yeast	0.64		0.55	131.1
	0.5 " " " autoclaved	0.61		0.36	130.2
	0.5 " " " "	0.56		0.21	108.9
	pH 10				
	Control	0.16		0.07	47.4

\* After 4 months, the butter yellow concentration was 0.28 mg. per gm. of diet; the iodine number 121.6.

† After 4 months, the butter yellow concentration was 0.38 mg. per gm. of diet; the iodine number 123.6.

‡ After 4 months, the butter yellow concentration was 0.20 mg. per gm. of diet; the iodine number 123.0.

§ After 4 months, the butter yellow concentration was 0.21 mg. per gm. of diet; the iodine number 125.7.

brewers' yeast, of aqueous extracts of yeast (Natuplex B, Squibb), crude extracts of liver, molasses, and milk sugar residue. It is of interest that a liver fraction, soluble in 95 per cent alcohol, exhibited less antioxidant properties than that of crude extracts or of Lederle's liver fraction (a preparation representing a concentrate of the antipernicious anemia factor).<sup>4</sup>

<sup>4</sup> The liver fraction, soluble in 95 per cent alcohol, and the concentrate of the antipernicious anemia factor were kindly furnished by Dr. D. Klein of The Wilson Laboratories, Chicago, and by Dr. Y. Subbarow of the Lederle Laboratories, Inc., Pearl River, New York, respectively.

High antioxidant potency was found in a purified, but still very crude concentrate, of folic acid,<sup>5</sup> known as the norit eluate factor (5).

Autoclaving of whole yeast resulted in definite destruction of antioxidant activity regardless of the pH during the heating process. Unlike whole yeast and rice bran extract, the antioxidant of aqueous yeast extract (Natuplex B) proved to be heat-resistant, at least under existing experimental conditions.

TABLE IV

*Antioxidant Activity of Sources of B Complex Other Than Rice Bran Extract and Brewer's Yeast*

Experiment No.	Source of B complex added to basic diet	Butter yellow per gm. diet			Iodine No. (21st day)
		5th day	8th day	21st day	
1	1.0 cc. Valentine's crude liver extract	0.55	0.48	0.38	131.0
	0.2 " " " " "	0.48	0.43	0.40	136.0*
	0.01 " " " " "	0.43	0.32	0.19	120.2
	Control (no supplement)	0.27	0.07	0.07	
2	1.0 cc. Lederle's crude liver extract	0.58	0.53	0.41	134.3
	Control	0.23	0.10	0.06	43.8
3	1.0 cc. Wilson's purified liver extract (fraction of aqueous liver extract soluble in 95% alcohol)	0.31	0.15	0.11	51.2
4	Control	0.31	0.17	0.07	
	1.0 cc. solubilized liver extract (norit eluate factor)		0.53	0.48	132.1
	Control	0.31	0.26	0.08	70.5
5	0.2 cc. molasses	0.46	0.50	0.35	126.2
	0.04 " "	0.38	0.27	0.06	52.9
6	0.3 gm. milk sugar residue				
	Batch 1	0.55	0.46	0.08	55.6
	" 2	0.56	0.45	0.08	57.4
	Control	0.29	0.19	0.07	52.9

\* After 4 months, the butter yellow concentration was 0.06 mg. per gm. of diet; the iodine number 47.7.

*Sulfonamide Drugs and p-Aminobenzoic Acid As Antioxidants*—The catalytic oxidation of *p*-aminobenzoic acid, pyrogallol, and tyramine by peroxidase is inhibited by sulfanilamide (6). The experiments presented in Table V are tests carried out with sulfanilamide and sulfaguandine for similar antioxygenic activity when studied in the absence of any enzyme in the linoleic acid-butter yellow system. The possible reversal of the reac-

<sup>5</sup> Received through the courtesy of Dr. C. A. Elvehjem, University of Wisconsin, Madison.

tion by *p*-aminobenzoic acid was also investigated. Whereas only a slight antioxidant effect was found with the sulfonamide drugs, *p*-aminobenzoic acid at a level of 50 mg. per 10 gm. of the experimental ration afforded considerable protection against oxidation. A synergistic effect of either sulfanilamide (0.5 gm.) or sulfaguanidine (0.5 gm.) with *p*-aminobenzoic acid (20 mg.) was observed when used in combined doses (Experiment 2, Table V).

*Antioxidant Activity of Individual B Vitamins*—In order to study the antioxidant activity of all the known B vitamins as a group they were added to 10 gm. of the experimental mixture (corn-starch, linoleic acid, and

TABLE V  
*Antioxidant Activity of Sulfonamide Drugs and p-Aminobenzoic Acid*

Experiment No.	Supplement to basic diet	Butteryellow per gm. diet			Iodine No. (21st day)
		5th day	8th day	21st day	
		mg.	mg.	mg.	
1	0.5 gm. sulfaguanidine	0.15	0.11	0.11	60.0
	0.5 " " + 50 mg. <i>p</i> -aminobenzoic acid	0.54	0.48	0.44	128.2
	0.5 gm. sulfanilamide	0.21	0.24	0.15	87.2
	0.5 " " + 50 mg. <i>p</i> -aminobenzoic acid	0.58	0.48	0.44	132.0
	Control	0.12	0.07	0.06	59.6
2	0.5 gm. sulfaguanidine	0.11	0.10	0.11	68.2
	0.5 " " + 20 mg. <i>p</i> -aminobenzoic acid	0.39	0.33	0.32	124.1
	0.5 gm. sulfanilamide	0.17	0.15	0.15	69.4
	0.5 " " + 20 mg. <i>p</i> -aminobenzoic acid	0.45	0.41	0.37	126.0
	50 mg. <i>p</i> -aminobenzoic acid	0.49	0.50	0.46	132.5
	20 " " "	0.30	0.18	0.08	64.2
	Control	0.10	0.06	0.05	60.0

butter yellow) in amounts at least equal to the optimal rat-day doses; *i.e.*, thiamine 50  $\gamma$ , riboflavin 200  $\gamma$ , pyridoxine 50  $\gamma$ , calcium pantothenate 100  $\gamma$ , biotin 20  $\gamma$ , inositol 1 mg., *p*-aminobenzoic acid 1 mg., nicotinic acid 1 mg., and 25 mg. of choline chloride. At these levels no inhibition of the oxidation of linoleic acid was observed. When in the experiments with sulfonamide drugs higher doses of *p*-aminobenzoic acid (20 mg. or above) proved to have antioxygenic activity, the investigations with B vitamins were revised by using 20 times the amounts previously tested. To this mixture were added also 250  $\gamma$  of a concentrate of folic acid, with a purity of 5.5 per cent.<sup>6</sup> The B vitamins at these higher levels were tested, combined

<sup>6</sup> Kindly supplied by Dr. R. J. Williams, The University of Texas, Austin.

and singly. With the exception of *p*-aminobenzoic acid none of the vitamins showed any significant antioxidant activity. Nicotinic acid and the preparation of folic acid gave very slight and transitory protection that lasted in both cases only to the 5th day of the experiment. The activity of the combined B vitamins was determined in large part by the presence of *p*-aminobenzoic acid. Removal of *p*-aminobenzoic acid deprived the mixture of significant antioxygenic activity, although minor and very transitory activity could be demonstrated even in the absence of *p*-aminobenzoic acid.

*Hydroquinone and  $\alpha$ -Tocopherol in Stabilization of Linoleic Acid-Butter Yellow System*—Hydroquinone (7) and  $\alpha$ -tocopherol (8) are known to possess antioxidant properties. For purposes of comparison it was desirable to measure their ability to stabilize the linoleic acid-butter yellow system. 100 mg. of hydroquinone added to 10 gm. of the experimental mixture assured complete protection even after 21 days (iodine number 132.0 and concentration of butter yellow 0.59 mg. per gm.). Smaller amounts of hydroquinone (20 mg.) reduced the protection so that after 21 days the iodine number was 124.2 and the concentration of butter yellow 0.37 mg. per gm.

$\alpha$ -Tocopherol was found to be less potent as an antioxidant than was hydroquinone. 25 mg. of synthetic  $\alpha$ -tocopherol (Merck), when tested in the usual manner, showed complete protection up to the 8th day only, the concentration of butter yellow at this time being 0.60 mg. per gm. By the 21st day, however, the value fell to 0.16 mg. per gm. and the iodine number was 74.1.

## DISCUSSION

Although the importance of antioxidants as means for retardation of autoxidation has long been recognized (9) in the field of chemical technology including food conservation, their part in strictly biological processes has not yet received the attention it seems to merit. Only quite recently has discussion started on the rôle of special antioxidants, mainly of vitamin E ( $\alpha$ -tocopherol) as intestinal antioxidants with their point of attack in the intestinal lumen. The relevant claims dealt with the intestinal destruction of ingested carotene in the presence of unsaturated fatty acids and in simultaneous absence of vitamin E (10-12). Supplements of vitamin E prevented the coupled intestinal oxidation of carotene.

Past (1) and present studies have extended the scope of study on antioxidants beyond that of vitamin E and generally beyond the class of purely fat-soluble antioxygenic substances (13), and as far as coupled oxidations are concerned, beyond carotene to other autoxidizable substances such as butter yellow.

From the present investigations it became apparent that  $\alpha$ -tocopherol and the classical antioxidant, hydroquinone, are not as potent antioxidants for the preservation of butter yellow as are grains, yeast, liver, and their crude aqueous extracts. The common characteristic of all these and other similarly active substances (molasses, milk sugar residue) as well as concentrates is their occurrence in crude sources of the whole vitamin B complex. Of all the known members of the B complex, however, only *p*-aminobenzoic acid exhibited appreciable antioxidant activity. This finding notwithstanding, one can definitely conclude that *p*-aminobenzoic acid is not identical with the potent antioxidant as it occurs in sources of the B complex and especially with that in rice bran extract or in solubilized liver (norit eluate factor (5)).

With the norit eluate factor and the rice bran extract one is confronted with the same situation regarding the neutralizing power of the eluate factor and *p*-aminobenzoic acid on the toxic effect of sulfaguanidine in rats. It has recently been shown (5) that this liver factor is distinct from *p*-aminobenzoic acid for various reasons, the most important of which is the negative result of the microbiological assay for *p*-aminobenzoic acid in therapeutically effective amounts of the eluate factor. In our own investigations crude rice bran extract (with about 70 per cent dry residue) was weight for weight (50 mg.) definitely more active as an antioxidant than was *p*-aminobenzoic acid. Furthermore, in microbiological assay crude rice bran extract was found to be almost devoid of free *p*-aminobenzoic acid (1  $\gamma$  per gm.<sup>7</sup>).

Against the identity of the antioxidant in rice bran extract with folic acid militates the fact that the microbiological assay of rice bran extract revealed the presence of only about 6  $\gamma$  of folic acid per cc.<sup>7</sup> 250  $\gamma$  of a folic acid concentrate with a content of about 13  $\gamma$  of pure folic acid exhibited an incomparably lower antioxygenic activity than has been exerted by 0.33 or even 0.07 cc. of rice bran extract. In this connection it is of interest to note that through recent investigations of Nielsen and Elvehjem (14) the identity of the liver factor which neutralizes the growth-depressing effect of succinylsulfathiazole in rats with folic acid became very probable.

Aromatic amines, such as aminophenols, are commonly used antioxidants in technical chemistry, for instance in preservation of rubber or oil (9). In view of this fact and of the close structural similarity the antioxidant quality of *p*-aminobenzoic acid could almost have been predicted. The biological consequences of the rôle of *p*-aminobenzoic acid as an antioxidant invite further investigations.

<sup>7</sup> We are indebted for this information to Dr. M. Landy of the Research Laboratories, the S. M. A. Corporation, Chagrin Falls, Ohio.



## SUMMARY

1. The coupled oxidation of butter yellow proceeds more slowly in the heterogeneous system consisting of corn-starch, methyl linoleate, and butter yellow than in a system in which linoleic acid is substituted for its methyl ester.
2. Grains (oats, wheat, corn) and the commercial oat flour preparation Avenex exhibited high antioxidant activity in the linoleic acid-butter yellow system.
3. Aqueous rice bran extract contains a very potent antioxidant. Other sources of the vitamin B complex such as yeast, yeast extract, liver extract, molasses, and milk sugar residue have also shown antioxygenic properties.
4. The antioxidants in rice (polished or unpolished), rice bran extract, Avenex, and yeast have been found to be heat-labile. In yeast extract or in rice bran extract no destruction by autoclaving at pH 10 was observed.
5. The antioxidant of rice bran extract is dialyzable and partially soluble in linoleic acid.
6. Of all the known B vitamins only *p*-aminobenzoic acid has proved to be significantly antioxygenic, at least under the experimental conditions chosen.
7. The antioxidant of rice bran extract and of potent liver fractions, however, is certainly not identical with *p*-aminobenzoic acid.
8. Vitamin E and hydroquinone are only moderately active antioxidants for the system under investigation.

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# ON THE ALCOHOL SOLUBILITY OF PROLACTIN

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The various methods of extracting and purifying prolactin, the lactogenic hormone of the anterior pituitary gland, are based at least in part on the observation that this hormone is soluble in 60 per cent ethanol (pH 10) and 85 per cent acetone (pH 1.5), while most of the accompanying inert proteins are not (1-4). Alcohol and acetone of higher concentration, however, will precipitate the hormone from these solutions.

In the course of experiments on the purification of prolactin the surprising observation was made that this protein hormone becomes highly soluble in 99.8 per cent methanol and 95 per cent ethanol as well as in certain other organic solvents if the pH is adjusted appropriately. In view of the fact that up to date only a few alcohol-soluble proteins have been described it was thought desirable to learn more about this unusual behavior of prolactin. In the present report the results of investigations with purified hormone preparations will be presented first, followed by a description of experiments with fresh pituitary glands.<sup>1</sup>

## EXPERIMENTAL

*Purified Prolactin and Organic Solvents*—Prolactin, though insoluble in organic solvents at its isoelectric point of pH 5.7, is highly soluble in 99.8 per cent methanol and 95 per cent ethanol in the presence of a small amount of acid. Under the same condition it is also dissolved, but to a lesser degree, by absolute ethanol and propylene glycol. The most favorable acidity apparently lies in the neighborhood of pH 3; yet the solubility of prolactin in alcohols extends over a wide range, from below pH 1 to about pH 4.7. If the acidity is increased much beyond pH 1, the hormone becomes reversibly insoluble in these solvents as it does in water. Hydrochloric, sulfuric, glacial acetic, sulfosalicylic, trichloroacetic, and probably many other acids may be used to bring prolactin into solution with methanol or 95 per cent ethanol. Sulfosalicylic and trichloroacetic acids deserve special attention, for prolactin is precipitated completely by these two protein precipitants from an aqueous solution. Other excellent solvents for prolactin are anhydrous acids such as glacial acetic and propionic acids. The following studies, however, will be confined to solutions of prolactin in methanol and ethanol.

<sup>1</sup> For the biological assays see the following paper.

The isolation of prolactin from its solution in acid alcohols can be achieved in three different ways.

*Precipitation with Ether or Acetone*—Though both solvents will precipitate most of the active material when added in quantities equal to that of the methanol solution, it was found more satisfactory to use 2 volumes of technical ether or 4 volumes of acetone, because in that case the precipitation flocculates and settles immediately. By decanting off the clear supernatant and washing the precipitate two or three times with dry ether, before it is brought to dryness, white powders are obtained which are again completely soluble in methanol without any further addition of acid.

*Precipitation by Raising pH to 5 or Above*—The advantage of this method lies in the smaller volume used; the disadvantages are, however, that any pigment present will precipitate along with the hormone and that in order to bring such preparations back into methanol solution the pH has to be readjusted. Elimination of the pigment can be achieved by the use of charcoal (norit A, Eimer and Amend). As large amounts of it adsorb lactogenic activity, one has to add the charcoal in small portions to the acid methanol solution of prolactin until a sample upon filtration is free from pink color. In a number of experiments carried out with different starting materials and under varying conditions, it was always found satisfactory to use 0.5 to 0.6 gm. of norit A per 1 gm. of dissolved protein, regardless of whether the concentration of protein in methanol was 0.08 or 1.6 per cent.

*Evaporation of Solvent in Vacuo at Low Temperature*—It was found that evaporation of the solvent is possible without loss of any prolactin activity. Small amounts can even be carefully evaporated on the water bath. Since a methanol solution of prolactin may be filtered through a Seitz pad EK without any loss of potency, sterile solutions of prolactin in methanol can thus be prepared and afterwards evaporated under sterile conditions.

In order to obtain some information on the extent to which prolactin is soluble in both methanol and ethanol, a number of solubility tests were made with two highly purified prolactin preparations, one derived from sheep, the other from beef pituitary glands. A description of these two preparations follows.

A new method, which will be outlined in the following communication, was applied to obtain hormone preparations assaying approximately 30 i.u. per mg. They were completely soluble in alcohol but still contained some salt. They were, therefore, dialyzed in the ice box, until all of the material had precipitated isoelectrically (12 days). The precipitate was dissolved in methanol with the addition of a small amount of concentrated hydrochloric acid. 2 volumes of ether were added and the resulting precipitate was separated by centrifuging and was then dried with ether.

The dried preparations were subsequently twice redissolved in methanol and precipitated with ether in order to get rid of a small amount of material which was less soluble in methanol. No acid was used in these last steps. The two preparations thus obtained were dried in a desiccator over calcium chloride. Biological assays indicated that both preparations now contained only 20 i.u. per mg. As may be seen from the experiments discussed in this and the following publication, treatment with acid methanol does not change the potency of prolactin preparations. It is therefore suggested that this reduction in potency is due to the prolonged dialysis rather than to the treatment with acid methanol.

When 25.0 mg. of each preparation were dissolved in 10 cc. of water, the solutions showed pH values of 3.04 and 3.10 for sheep and beef prolactin, respectively. (The same values were encountered when methanol was used as solvent.) To get the isoelectric reaction (pH 5.70), 2.63 and 2.58 cc. of 0.01  $\text{N}$  sodium hydroxide had to be added. This amounts to 1.04 milliequivalents of hydrochloric acid bound in each gm. of prolactin hydrochloride, corresponding to 1.46 per cent of basic nitrogen. Chlorine determinations, on the other hand, revealed 3.96 and 4.08 per cent chlorine, respectively, corresponding to an average of 1.58 per cent of basic nitrogen.

To 0.200 gm. of prolactin hydrochloride (sheep) 0.8 cc. of methanol (99.5 per cent, Eimer and Amend) was added and the mixture stirred well with a glass rod. An extremely viscous solution formed which became somewhat opalescent while it was being shaken at room temperature for 4 hours. It was then centrifuged for 30 minutes at 2000 R.P.M. which caused separation of a small amount of clear heavy liquid from the gelatinous remainder. The supernatant solution was weighed, brought to dryness in a vacuum desiccator over calcium chloride, and the dry weight determined. It was found that 56.2 mg. of methanol solution contained 12.3 mg. of prolactin. The concentration was, therefore, 21.9 per cent by weight ( $t = 24.5^\circ$ ).

Other tests were performed to determine the solubility of the two prolactin preparations in absolute and 95 per cent ethanol as well as in methanol saturated with sodium chloride (1.29 per cent of sodium chloride by weight). All these experiments were made by extracting 50 mg. of prolactin hydrochloride with 0.4 cc. of the solvent for 4 hours and evaporating a determined weight of the centrifuged solution to dryness. The results of these experiments are summarized in Table I. Though saturation was reached with certainty only in three experiments, others are listed as well to show the minimum solubilities. From the data in Table I, the following can be seen. (1) Prolactin hydrochloride is quite soluble even in absolute ethanol. The preparation derived from beef pituitary glands exhibited a somewhat higher solubility. (2) The solubility in 95 per cent ethanol is

at least 3 times greater than in absolute ethanol. (3) Prolactin hydrochloride is highly soluble in methanol even in the presence of sodium chloride.

*Acid Alcohol Extraction of Prolactin from Fresh Pituitary Glands*—For the following experiments, fresh undissected pituitary glands derived either from sheep or from beef were extracted with enough acidified absolute ethanol or methanol so that a final concentration of approximately 95 per cent resulted.

250 gm. of undissected pituitary glands, passed two or three times through a meat grinder, are extracted in an ice bath for 1 to 2 hours with 4 liters of methanol and 5 cc. of concentrated hydrochloric acid (pH 3.9). If a stirrer that is not powerful enough is used, it might be necessary to force the tissue through a sieve, so as to avoid the formation of lumps. The mixture is then centrifuged for 10 minutes at 2000 R.P.M. The pink

TABLE I  
*Solubility of Purified Prolactin in Methanol and Ethanol*

pH 3.1,  $t = 24-25^\circ$ .

Solvent	Source	Solubility of prolactin, per cent by weight
Methanol, 99.5%...	Sheep	21.9
" 99.5% .....	Beef	>14.3
Ethanol, 100%...	Sheep	3.5
" 100% .....	Beef	4.4
" 95% .....	"	>12.9
Methanol, 99.5%, saturated with NaCl ..	"	>13.2

extract may be treated in either one of two ways. (a) 2 volumes of technical ether are added, causing an immediate precipitation which settles down within a few minutes. The clear supernatant is decanted and the remainder centrifuged. The solids are then stirred up in the centrifuge cup with dry ether and again centrifuged. They are removed from the cup with dry ether, collected on a Buchner funnel, and, while still ether-wet, transferred to a mortar and ground to complete dryness. The yield is about 10 gm. (b) The extract is stirred for a few minutes with 5 gm. of norit A, centrifuged, and freed from colloidal charcoal by filtration through a Seitz GP or EK filter pad. The filtrate, which is golden in color, is neutralized with 5 M NaOH to pH 5 to 6. This precipitate is worked up in the same manner as described above. The yield is about the same as in the first alternative.

In Table II the results of five experiments are recorded. The data indicate that methanol extracts from fresh pituitary glands contain about

two-thirds of the prolactin activity in the case of sheep and three-fourths in the case of beef. The yield of active material is somewhat lower with ethanol. In Experiment 13-hs ground glands were pulverized in a mortar with the addition of dry ice until all of it went through a 40 mesh sieve, but no change in the distribution of the hormone between solution and

TABLE II

*Acid Methanol Extraction of Prolactin from Fresh Pituitary Glands. Distribution of Weights and Activities between Alcohol Extracts and Residues*

All values are calculated on the basis of 1 kilo of whole pituitary glands.

Preparation No.	pH of methanol extract	Weight of fractions	Prolactin activity		Total activity
			Per mg.	Total	
Sheep pituitary glands					
		gm.	i.u.	i.u.	per cent
4-hs-4, methanol-soluble* . . . . .	2.5	43.8	1.85	81,000	63.9
4-hs-2, methanol-insoluble* . . . . .		158.0	0.29	45,800	36.1
				126,800	100.0
3-hs-4, ethanol-soluble* . . . . .	2.8	40.9	1.63	66,700	50.5
3-hs-2, ethanol-insoluble* . . . . .		152.0	0.43	65,400	49.5
				132,100	100.0
13-hs-4, methanol-soluble . . . . .	1.7	42.0	3.44	144,500	67.9
13-hs-2, methanol-insoluble . . . . .		145.5	0.47	68,400	32.1
				212,900	100.0
Beef pituitary glands					
6-hb-4, methanol-soluble† . . . . .	3.9	37.0	2.30	85,100	74.1
6-hb-2, methanol-insoluble† . . . . .		148.8	0.20	29,800	25.9
				114,900	100.0
5-hb-4, ethanol-soluble† . . . . .	3.2	32.1	2.60	83,500	72.1
5-hb-2, ethanol-insoluble† . . . . .		161.6	0.20	32,300	27.9
				115,800	100.0

\* The same lot of sheep pituitary glands was used.

† The same lot of beef pituitary glands was used.

residue was found. As can be seen from Table II, the potency of the active fractions lies between 1.6 and 3.4 i.u. per mg. Since purest prolactin assays 30 i.u. per mg., these crude fractions contain between 88.5 and 94.6 per cent impurities, including 2.6 to 3.2 per cent ash.

As can be seen from Table II, crude prolactin preparations with only 2 to 3 i.u. per mg., containing probably between 80 and 90 per cent of inert

proteins, are easily soluble in acidified 95 per cent methanol or ethanol. It must, therefore, be concluded that the solubility in alcohols is a much more common property of proteins than had up to now been realized.<sup>2</sup>

The results reported in Table II show that a considerable part of the prolactin activity remains in the acid methanol-insoluble residue. The same observation has been made with other extraction methods, as can be seen from experiments recorded in Table III. In Experiments 9-ls and 37-pr the fresh sheep pituitary glands were extracted with water at an alkaline pH. (Different lots of pituitary glands were used for the three experiments listed.)

Experiments designed to increase the extraction of prolactin activity were not successful. Even if it is assumed that the determination of the prolactin activity in the residues is falsified by the presence of inert pro-

TABLE III

*Comparison of Aqueous and Alcoholic Extractions of Prolactin*

All values are calculated on the basis of 1 kilo of whole sheep pituitary glands.

Experiment No.	Description of extraction			Prolactin	
	Type	pH	Total time	In residue	In extract
9-ls	Water, 2 extractions	9.6	4	42,100	71,400
		10.5			
37-pr	" 3 "	9.7	23	57,500	147,900
		9.9			
		10.2			
59-pr	Methanol, 1 extraction	2.0	1½	49,000	81,600

teins (5), the question why part of the lactogenic potency resists extraction remains unanswered.

Since the greater portion of crude prolactin fractions is soluble in alcohols, a further treatment with these solvents cannot be expected to increase their purity. However, if a 2 per cent aqueous solution of the material is precipitated at pH 2.8 to 3.6 with 2 per cent sodium chloride and the precipitate treated again with methanol, preparations containing up to 15 i.u. per mg. are obtained.

2 gm. of the material are dissolved in 75 cc. of water. If necessary, the pH of the aqueous solution is adjusted to 2.8 to 3.6. After the mixture

<sup>2</sup> The question whether other pituitary hormones are contained in the methanol extracts from fresh pituitary glands can be answered at this time only with regard to the follicle-stimulating hormone. It was found that follicle-stimulating hormone is not soluble in 95 per cent methanol under the experimental conditions described above.

has been stirred in an ice bath for 1 hour, a solution of 2 gm. of sodium chloride in 25 cc. of water is added and stirring continued for 15 minutes. It is then centrifuged. The supernatant is discarded, and the residue is extracted twice with 20 cc. of methanol to which 1 drop of concentrated hydrochloric acid is added. The methanol extractions are combined and precipitated with 80 cc. of ether. The solids from the ether precipitation are worked up in the same way as described for the crude preparations. The yield is 0.20 to 0.25 gm. of material, assaying 13 to 16 i.u. per mg.

In Table IV the distribution of weights and activities between the methanol-soluble and methanol-insoluble fractions of the precipitate obtained with 2 per cent aqueous sodium chloride is shown by two examples.

TABLE IV

*Purification of Crude Prolactin Fraction by Salt Fractionation and Methanol Extraction of Salt Precipitate*

All values are calculated on the basis of 1 kilo of whole pituitary glands.

Preparation No., 2% NaCl ppt.	pH of methanol extract	Weight of fractions	Prolactin activity		Total activity	
			Per mg.	Total		
Sheep prolactin derived from 42.0 gm. of Preparation 13-hs-4 (see Table II)						
67-pr-4, methanol-soluble	3.6	gm. 5.2	per cent 15.7	i.u. 15.7	i.u. 81,600	per cent 60.6
67-pr-2, methanol-insoluble		28.0	84.3	1.9	53,200	39.4
		33.2	100.0		134,800	100.0
Beef prolactin derived from 37.0 gm. of Preparation 6-hb-4 (see Table II)						
66-pr-4, methanol-soluble	2.8	3.7	13.2	12.9	47,700	53.5
66-pr-2, methanol-insoluble		24.4	86.8	1.7	41,500	46.5
		28.1	100.0		89,200	100.0

These data indicate that (1) the total yields from 1 kilo of whole sheep and beef pituitary glands were 81,600 and 47,700 i.u., respectively, (2) 2 per cent aqueous sodium chloride precipitated about 79 and 76 per cent of all proteins present in the original crude prolactin fractions, (3) not more than 16 per cent of the salted-out material was soluble in methanol. These fractions, however, contained 54 to 61 per cent of the prolactin activity.

It could be shown that this low methanol solubility was not a property of the original crude prolactin fractions (Preparations 13-hs-4 and 6-hb-4), the greater part of which was methanol-soluble. The great decrease in alcohol solubility of these proteins when wet salt precipitates were extracted with methanol, but not when the dry material was taken up in the same



solvent, suggests that alcohol denaturation is favored by the presence of either salt or water, or both.

#### DISCUSSION

Solubility of a protein in a high concentration of alcohol has been considered up to now to be a very rare phenomenon. It has been shown in the present communication that alcohol solubility is, on the contrary, quite a common property among native proteins. Prolactin constitutes but a small fraction of the 20 per cent of pituitary proteins which were found to be soluble in 95 per cent alcohol.

The known alcohol-soluble proteins are of two distinctly different types:

1. Proteins which are alcohol-soluble at neutral reaction. Only a few plant proteins, the prolamins, are known to show such a property. They are best soluble in 70 per cent alcohol, but insoluble in either pure alcohol or pure water. They are believed to owe their peculiar behavior to a deficiency in charged groups and to an abundance of uncharged polar groups (low basic but high amide nitrogen).

2. Proteins which are alcohol-soluble due to salt formation. In most instances the solubility of proteins in high concentrations of alcohol has been investigated only at a limited pH range. A protein which shows the remarkable property of being soluble in slightly ammoniacal 99 per cent ethyl alcohol is carbonic anhydrase, as was recently reported by Scott and by Scott and Fisher (6). A number of other proteins are known to be soluble in 60 to 70 per cent alcohol in the presence of alkali, one of them being prolactin (1). On the other hand, proteins which are soluble in such alcohol concentrations in the presence of acid are also known. To this group belongs Osborne's alcohol-soluble casein fraction with the surprisingly high molecular weight of  $375,000 \pm 11,000$  (Svedberg and coworkers (7)) and various hormones. Though prolactin is quite unique in its high affinity towards alcohols, it is probable that it differs from the known representatives of this group more in degree than in kind. In which respect these proteins differ from other proteins which are unable to form alcohol-soluble salts is not known. Osborne and Wakeman (8) were unable to attribute the alcohol solubility of their protein to any special groups in its molecule, especially if comparison was made with the alcohol-insoluble casein; both have approximately the same proportion of amide nitrogen, and casein contains about 36 per cent more basic nitrogen than Osborne's protein. However, the observation that alcohol-soluble pituitary proteins, except prolactin, lose their solubility in methanol during the process of purifying prolactin<sup>3</sup> suggests that the relative proportion of amino acids is not the only decisive factor which determines the solubility, but that the general structure of a protein is also of the greatest importance.

<sup>3</sup> See also the following paper.

## SUMMARY

Prolactin is shown to be highly soluble, at a pH below its isoelectric point, in 99.8 per cent methanol and 95 per cent ethanol. This peculiarity, however, is shared by a large part of the proteins from fresh pituitary glands.

A method is described for the extraction and purification of prolactin.

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# A NEW METHOD FOR THE PREPARATION OF PROLACTIN

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Among the numerous methods for the extraction and purification of the hormones of the anterior pituitary recorded in the literature, several procedures have been worked out especially with the view of securing as many as possible of the active principles (1-3). However, considerable overlapping of the active principles in the fractions obtained makes the preparation of the hormones in pure form quite laborious. In view of these shortcomings we have tried to work out a method in which the starting material would be fractionated in such a way that a preparation of several hormones from one batch of glands could be more easily undertaken.

Among the different methods tried, the emulsification of the pituitary proteins with organic solvents such as chloroform and their subsequent separation as a gel proved to be most promising.

This method of precipitating proteins by treating their aqueous solution with chloroform has been known for a number of years and has been used frequently in the purification of polysaccharides (4). The only application of this method for the purification of a hormone was made by Gurin, Bachman, and Wilson (5), who used it to remove traces of protein from a highly purified chorionic gonadotropin preparation. The possibility of using this method for a separation of different protein fractions was, however, not indicated by these authors.

When macerated, undissected pituitaries are shaken with chloroform at a pH of between 5 and 6 and subsequently centrifuged, three distinct layers are formed. The lowest consists of chloroform in which most of the lipid substances of the tissue are dissolved. The layer above the chloroform is a gel which contains the bulk of the tissue proteins, together with prolactin and adrenotropic hormone. The top layer is a clear aqueous solution containing the gonadotropic hormones, thyrotropin, pituitrin, and other easily soluble substances.

## EXPERIMENTAL

*Biological Assays*—The prolactin activity of our preparations was determined by the weight increase of the crop sacs of white Carneau<sup>1</sup> pigeons

<sup>1</sup> The pigeons were the same breed as those used by Dr. Riddle for the dose-weight curve.

6 weeks of age according to the Riddle, Bates, and Dykshorn dose-weight curve (6). Care was taken to check all crop sacs for local stimulation. Assays showing no local response were rejected as negative. For the conversion of Riddle units into international units, at least one group of squabs was injected each week with a standard, the potency of which had been determined by comparison with an international standard of prolactin.<sup>2</sup> Five to ten pigeons were used per group.

1. *Chloroform Method*—1 kilo of fresh, frozen, undissected pituitaries (sheep or beef or hog) was passed through the meat grinder two or three times. 1 liter of cold water was added and the pH kept between 8 and 9 by the addition of 5 N sodium hydroxide. The mixture was stirred in an ice bath for 4 hours. 2 liters of water and 2 liters of chloroform were then added and enough glacial acetic acid to adjust the pH to a point between 5 and 6; i.e., near the isoelectric point of prolactin (pH 5.70). Stirring was continued for another hour. The mixture was then centrifuged for 25 minutes at 2000 R.P.M., resulting in the formation of three layers.

The clear, red-colored aqueous supernatant was decanted and poured into 5½ times its volume of acetone, forming a white precipitate which contained the gonadotropic and thyrotropic hormones. The yield was approximately 20 gm. for sheep pituitary, less for beef and hog pituitaries. About 70 per cent of the original chloroform used could be decanted by lifting the solid cake. The latter was then stirred with an excess of acetone, filtered, and washed with more acetone. It was dried, ground in a ball mill, and sifted through a 100 mesh sieve. The total yield was 175 to 180 gm., independent of the source of the pituitaries.

2. *Extraction of Prolactin from Chloroform Gel. Acid Methanol Extraction*<sup>3</sup>—40 gm. of the dried and sifted chloroform gel were extracted for 1 hour with 600 cc. of methanol (technical, 99.8 per cent), while enough concentrated hydrochloric acid was added (approximately 4 cc.) to keep the pH between 1.6 and 2.4 as measured by a glass electrode. Stirring was carried out in an ice bath. The residue, after centrifugation, was stirred up shortly with 400 cc. and then with 200 cc. of methanol without further addition of hydrochloric acid. The three methanol solutions were combined, adjusted to pH 3 with a few drops of 5 N sodium hydroxide, and poured into 2 volumes of technical ether. The white prolactin precipitate settled promptly and was centrifuged, washed with dry ether, and dried. The yield was 3 to 4 gm. The methanol-insoluble residue was dried with acetone.

3. *First Salt Fractionation*—3 gm. of the precipitate obtained from the

<sup>2</sup> We wish to thank Dr. Riddle for his courtesy in letting us have some standard preparations and also for assaying some of our material in his laboratory.

<sup>3</sup> For a discussion of the solubility of prolactin in acid methanol, see the preceding paper.

acid methanol extracts were dissolved in 150 cc. of water, the pH of the solution being kept at about 2.5 by dropwise addition of concentrated hydrochloric acid. Solid sodium chloride was added, with stirring, to a concentration of 5 per cent, causing prompt flocculation of the prolactin. The precipitate, after centrifuging, was extracted in a Waring blender with 150 cc. of methanol and centrifuged. The clear methanol supernatant was precipitated with 2 volumes of ether and the resulting prolactin precipitate dried. The yield was about 1 gm. The methanol-insoluble residue was reextracted in a Waring blender with 150 cc. of methanol, centrifuged, and the supernatant precipitated with ether, yielding 0.2 to 0.4 gm. of material which was somewhat less active than the precipitate from the first extract.

4. *Second Salt Fractionation*—The two methanol-soluble fractions obtained in step (3) were combined and again dissolved in water to a concentration of 2 per cent. Solid sodium chloride was added to a concentration of 2 per cent, and the resulting precipitate extracted with 80 cc. of methanol. The active fraction was then precipitated from this extract by neutralization with sodium hydroxide to pH 5 to 7. By this procedure the precipitation of any sodium chloride which is present in the methanol extract is avoided. The final precipitate was redissolved in 50 cc. of acidified methanol and reprecipitated with 2 volumes of ether. The yield from sheep pituitaries was 0.4 to 0.5 gm. of prolactin, assaying approximately 30 i.u. per mg.

A representative fractionation of sheep, beef, and hog pituitaries is recorded in Table I. The yields are calculated per kilo of fresh, undissected glands. It should be mentioned that, in agreement with the observation of Friedman and Hall (7), we found that different shipments of pituitaries of the same species vary considerably in prolactin activity.

As can be seen from Table I, the chloroform gel from sheep pituitaries contains approximately twice as much prolactin as that from beef and hog pituitaries. It is realized that the large amount of insoluble tissue proteins in the starting material may increase the titer of these fractions owing to delayed absorption (7). In a few instances in which the chloroform gel was compared with acetone-dried pituitaries prepared from the same shipment, the dried glands appeared slightly more potent. The acid methanol extraction of the chloroform gel (step (2)) increases the activity per mg. 8- to 10-fold, and a yield of 50 to 60 per cent of prolactin activity is obtained. The residue, containing about 40 per cent of total protein, shows an activity of 0.3 i.u. per mg. or about 10 per cent of the total activity. Again, the large amounts of inert protein may interfere with the determination of the fractions. The precipitation of prolactin from the supernatant with 5 per cent sodium chloride followed by extraction with ether increases the activity per mg. 2- to 3-fold, and a

TABLE I  
*Purification of Prolactin*

Fraction	Sheep pituitary			Beef pituitary			Hog pituitary		
	Activity	Yield per kilo	i.u. per kilo	Activity	Yield per kilo	i.u. per kilo	Activity	Yield per kilo	Activity per kilo
Step (1), chloroform gel. . . . .	i.u. per mg. (11-ls-2)* 1.18	173	183,700	i.u. per mg. (7-9-lb-2) 0.59	177	94,500	i.u. per mg. (16-lp-2) 0.45	178	80,000
Step (2), acid methanol extract; ether ppt. . . . .	(59-pr) 8.0	12.2	97,600	(52-pr) 5.0	11.5	57,500	(109-pr) 3.82	11.2	42,900†
Step (3), 5% NaCl ppt.; metha- nol-soluble fraction. . . . .	(81-pr) 15.6	4.6	72,000	(82-pr) 13.9	3.97	55,100	(110-pr) 8.98	6.44	57,800
Step (4), 2% NaCl ppt.; metha- nol-soluble fraction . . . . .	(83-pr) 30.2	1.78	53,900	(81-pr) 30.2	1.42	42,800	(111-pr) 19.0	2.86	54,400

\* The preparation numbers are given in parentheses.

† The determination of the activity unfortunately could not be repeated and is evidently wrong.

with 2 per cent sodium chloride (step (4)) again more than doubles the potency per mg.

With the method as described in this communication we have repeatedly prepared prolactin containing 30 i.u. per mg. from sheep pituitaries in yields of approximately 2 gm. per kilo of fresh glands. The final product from beef pituitaries varies in weight between 1.0 and 1.5 gm. and assays 26 to 30 i.u. per mg. With hog pituitaries, the method outlined brings the potency only to about 20 i.u. per mg. and necessitates further purification. All these preparations are amorphous, including those assaying 30 i.u. per mg. No physicochemical data concerning the purity of our preparations have been ascertained.

#### DISCUSSION

Several methods for the preparation of the lactogenic hormone from the anterior pituitary have been described in the literature, based on the extraction of the active principle with 60 per cent ethyl alcohol at pH 10 (8) and with 85 per cent acetone at pH 1.5 (9). Li, Lyons, and Evans (10) have obtained a pure protein of constant solubility and electrophoretic homogeneity, assaying 30 international prolactin units per mg. By a procedure based on Lyons' method, White, Bonsnes, and Long (11) have prepared a similar prolactin from which they obtained the hormone in crystalline form.

We have carried out some experiments trying to separate the gonadotropic hormones from prolactin by the use of protein precipitants such as sulfosalicylic and trichloroacetic acids. However, the high acidity of the solutions resulted in considerable losses of gonadotropic activity and the experiments were therefore abandoned. The great advantage of using the chloroform method in the preparation of prolactin is seen in the possibility of preserving the other pituitary hormones in such a form that their subsequent isolation can be easily undertaken. Furthermore, the method as described here allows the preparation of prolactin in practically pure form in yields higher than those previously reported (11).

The mechanism of the interaction between proteins and chloroform is not fully understood at present. Sevag and coworkers (12) are of the opinion that the protein when treated with chloroform does not undergo profound changes. By the use of this method, preparations of catalase (13), phosphatase (14), and of the immunologically active protein component of the streptococcal nucleoproteins (12) have been obtained without apparent denaturation. On the other hand, Andrews and coworkers (15), in their recent excellent study of the optimal conditions for the removal of proteins by chloroform, have come to the conclusion that "the precipitated protein is denatured by the process to the point of being insoluble in water, salt solutions, and dilute acid and alkali." Our experiments with



some of the active factors of the anterior pituitary, although not originally intended to contribute to this question, seem to indicate that the chloroform treatment does not impair the activity of the lactogenic hormone in the chloroform gel nor the potency of the follicle-stimulating hormone in the aqueous phase. We are inclined to believe that denaturation by chloroform treatment may depend, among other factors, on the pH, the concentration of the protein mixture, and the physical and chemical characteristics of the proteins involved.

#### SUMMARY

A method of treating fresh, macerated pituitaries with chloroform has been described. By this method the pituitary proteins are separated into two fractions, one which is soluble in water, containing among other active principles the gonadotropins and thyrotropin, and one which is a solid gel, containing prolactin and adrenotropin.

The preparation of prolactin from the chloroform gel by extraction with acid methanol and sodium chloride fractionation has been outlined. The final product of 30 i.u. per mg. is obtained in a yield of approximately 2 gm. from 1 kilo of fresh sheep pituitaries.

We wish to thank Dr. Karl Meyer of the College of Physicians and Surgeons, Columbia University, for his advice in our work and Dr. Eric Putter and Miss Frances Dobell of our Biological Laboratory for carrying out the biological assays.

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# THE SYNTHESIS OF PEPTIDES BY TRANSAMINATION

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The synthesis of proteins in biological systems is usually considered as a reversal of the enzymatic cleavage of these substances, the formation of peptide linkages between free amino and free carboxyl groups of different entities. In recent years Bergmann and his collaborators (1) have obtained experimental verification of this concept of peptide synthesis.

In spite of the recent rapid advances in our knowledge of the intermediary metabolism of proteins and amino acids and of the enzyme systems involved in these changes, little attention has been paid to the possibility that substances other than amino acids may take part in the biological synthesis of peptide chains. Linderström-Lang (2) has suggested a scheme for protein synthesis involving the reaction between  $\alpha$ -dicarbonyl derivatives and amines observed by Maurer and Woltersdorf (3) and enzyme systems such as the glutamic dehydrogenase of von Euler *et al.* (4) and the transaminases of Braunstein and Kritzmann (5).

Biological analogues of three chemical methods of converting  $\alpha$ -keto acids into amino acids have been demonstrated; namely, the reduction of the oximes of keto acids (6, 7), the hydrogenation of keto acids in the presence of ammonia (8, 4), and the transfer of amino groups from amino acids to keto acids (transamination) (9, 5). Several years ago we undertook to study in model systems the application of these methods to the conversion of certain  $\alpha$ -ketoacylamino acids into peptides (10). At that time we demonstrated the conversion, among others, of pyruvyl-*dl*-alanine into *dl*-alanylalanine by the catalytic reduction of its oxime and by hydrogenation in ammoniacal solution. Methods of preparing the ketoacylamino acids have been developed by Bergmann and Grafe (11).

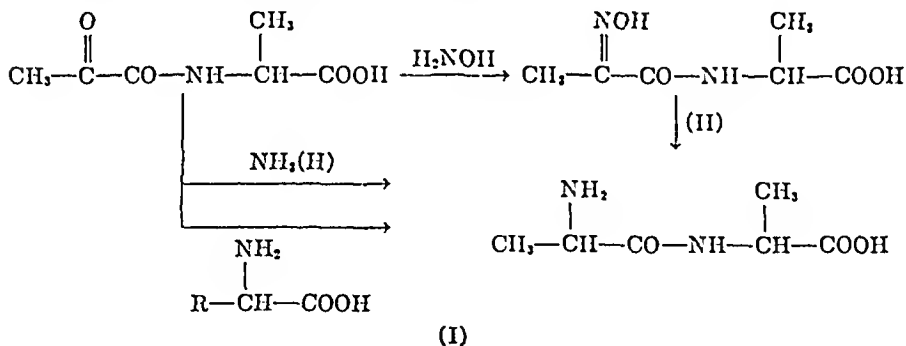
We have now completed the synthesis of alanylalanine from pyruvyl-alanine by transamination with  $\alpha$ -aminophenylacetic acid acting as donor of the amino group. The reaction was carried out by boiling an aqueous solution of pyruvyl-*dl*-alanine and  $\alpha$ -aminophenylacetic acid without a catalyst under the conditions previously employed in our laboratory for the study of uncatalyzed transaminations in model systems. Carbon dioxide and benzaldehyde were eliminated as volatile products. *dl*-Alanylalanine was isolated from the aqueous reaction mixture as the carbobenzoxy derivative. Since *dl*-amino acids were employed in the

reaction, the carbobenzoxyalanylalanine was obtained as a mixture of diastereoisomeric racemates which could be separated only with difficulty.

For purposes of comparison carbobenzoxyalanylalanine was prepared from alanine anhydride by a method analogous to that employed by Fischer and Kautzsch (12) in the preparation of benzoylalanylalanine. The procedure led to the formation of a mixture of diastereoisomers. By fractional crystallization the mixture could be separated into a number of fractions. Two of these, Fraction A melting at 144.5–145.5° and Fraction B melting at 168–169°, appeared to represent pure racemic modifications. The determination of their configurational relationship must await their resolution. In addition to these pure fractions a considerable amount of the total product remained in fractions melting below 140° and could be separated only with large losses of material. A small fraction, C, melting at 133.5–135°, resisted efforts at separation into the higher melting fractions. This material appeared to be either a solid solution of the other two fractions or a fortuitous mixture of constant melting point and crystalline appearance.

The carbobenzoxyalanylalanine obtained by transamination experiments gave on fractional crystallization a pure racemate melting at 167–167.5° and a second fraction melting at 133–135°. The latter fraction could not be separated into other fractions because of lack of material and the wastefulness of the process. Neither fraction showed a depression of the melting point when mixed with the appropriate fraction, B or C, obtained from alanine anhydride.

The three methods by which pyruvylalanine has been converted into alanylalanine are formulated as shown (I).

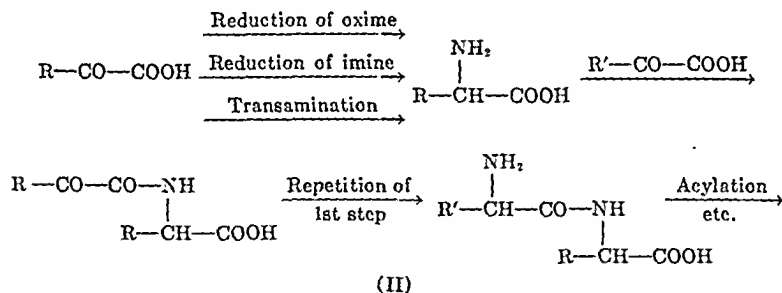


Although Braunstein and Kritzmann (13) and Cohen (14) have shown that simple peptides do not appear to act as amino group donors in transaminase-catalyzed systems, the possibility that pyruvylamino acids or analogous derivatives of other  $\alpha$ -keto acids may act as amino group acceptors in enzyme-catalyzed systems has not been investigated.

The results of our model experiments permit the postulation of a com-

plete scheme for the synthesis of peptide chains and proteins from non-nitrogenous precursors of the amino acids, the validity of which in biological systems should be considered. The nitrogen may be drawn from at least three sources, hydroxylamine, ammonia, or other amino acids. It will be observed that the scheme involves only two fundamental reactions, amination and acylation, both of which are known to take place in biological systems. By alternate repetition of these fundamental reactions a peptide chain of any conceivable length could be synthesized. The end-product would be limited only by the assortment of  $\alpha$ -keto acids available at the site of peptide synthesis.

The mechanism given in (II) is consistent with the results of Schoenheimer and his collaborators (15) in which isotopic nitrogen of a dietary amino acid was found in other amino acids of the body proteins. The protein molecule may be visualized as splitting at one peptide bond and the amino group so liberated may be removed by oxidative deamination, giving rise to an  $\alpha$ -ketoacyl polypeptide. Reamination of the latter, either by transamination or by a reductive amination, followed by reformation of the peptide linkage would lead to a protein containing a nitrogen atom from some other source.



This hypothesis for biological peptide synthesis would complement the classical conception of synthesis by the formation of peptide linkages from the amino acids already present in these systems. The two processes may be pictured as interlocking, so that certain stages in the synthesis of a given peptide chain may be accomplished by one process while other stages are accomplished by the second process. Which process would prevail at any given stage would depend entirely on the availability of the requisite intermediates as amino acids or as keto acids.

#### EXPERIMENTAL<sup>1</sup>

*dl*-Carbobenzoxylalanylalanine—An alkaline solution of alanylalanine was prepared from *dl*-alanine anhydride by the method of Fischer and Kautzsch

<sup>1</sup> All melting points reported are corrected.

(12). Alanine anhydride (8.1 gm.) was dissolved in 95 cc. of warm water. The clear solution was cooled to room temperature, treated with 65 cc. of normal aqueous sodium hydroxide solution, and allowed to stand at room temperature for 5½ hours. At this time the solution was chilled in an ice bath and with continuous stirring and cooling was treated with 72 cc. of normal sodium hydroxide and 12 cc. of benzyl chlorocarbonate added alternately, each in six equal portions, during 20 minutes. Vigorous stirring and cooling were continued for 20 minutes after the last addition. The slightly turbid reaction mixture was treated with decolorizing charcoal, filtered, and the clear filtrate made distinctly acid to Congo red by addition of concentrated hydrochloric acid. The product precipitated as an oil which quickly solidified on cooling and scratching. The yield of crude product after filtering by suction and washing with cold water was 10.5 gm. (63 per cent).

The crude material was subjected to fractional crystallization first from water, when it was observed that a considerable degree of separation could be attained by removing the material which crystallized on partial cooling of the hot solutions. A lower melting racemate which seemed to be present in larger quantity separated first from hot aqueous solutions. However, as this material was removed, a higher melting racemate was concentrated in the residual fractions. The latter appeared to have a lower solubility in water and separated first from hot solution upon recrystallization of the residual fractions. In this way about 2.3 gm. of fractions rich in the lower melting racemate, Fraction A, and 1 gm. of crude high melting racemate, Fraction B, were obtained. By applying a similar technique to intermediate fractions but using ethyl acetate as the solvent 1.5 gm. further, Fraction A-1, of the lower melting product were obtained. Recrystallization of the two fractions, A and A-1, from ethyl acetate and from water gave a total of 3.2 gm. of Fraction A, m.p. 144.5–145.5°, whose melting point remained constant and which gave no other fractions upon recrystallization from either solvent.

*Analysis*— $C_{14}H_{18}N_2O_5$ . Calculated, N 9.5; found, N 9.6, 9.5

The second crude racemate, Fraction B, melted at 154–156° and reached a constant melting point after seven recrystallizations from water. The yield of pure Fraction B, m.p. 168–169° with sintering at 165°, was 0.4 gm.

*Analysis*— $C_{14}H_{18}N_2O_5$ . Calculated, N 9.5; found, N 9.6, 9.7

Fraction A crystallized from water as a cottony mass of long, slender needles and from ethyl acetate as tufts of small needles. Fraction B crystallized from water as coarse, well defined needles. Both substances were easily soluble in methyl and ethyl alcohols, less soluble in cold ethyl

acetate (about 2 per cent) and cold water (about 1 per cent), and even less soluble in hot benzene, toluene, carbon tetrachloride, or ligroin. They could be precipitated in crystalline form by careful addition of ligroin to their solutions in ethyl acetate.

A certain amount of material accumulated in several small fractions, C, which crystallized from water as long needles, frequently in bunches, m.p. 133.5–135°. These fractions resisted separation into components even on crystallization from ethyl acetate-ligroin mixtures or from hot toluene in which they were slightly soluble at the boiling point.

*Analysis*— $C_{14}H_{15}N_2O_3$ . Calculated, N 9.5; found, N 9.5

One of the three fractions was probably a mixture of the other two, either as a molecular compound, a solid solution, or possibly a fortuitous mixture of such composition as to compensate differences in solubilities. To determine which fractions represented pure components, equal amounts (15 mg.) of each of different pairs (Fractions A and B, A and C) were mixed and recrystallized from water. Both mixtures melted over a wide range, Fractions A and B at 135–144° and Fractions A and C at 135–141°, indicating that, whereas Fractions A and B were probably pure racemates, Fraction C was either a solid solution or a mixture.

*Transamination with Pyruvylalanine and  $\alpha$ -Aminophenylacetic Acid*—Pyruvylalanine was prepared by the procedure of Shemin and Herbst (10). A solution of 3.18 gm. (0.02 M) of *dl*-pyruvylalanine in 200 cc. of water was boiled under a reflux with 3.02 gm. (0.02 M) of *dl*- $\alpha$ -aminophenylacetic acid for 23 hours in an atmosphere of nitrogen. The presence of carbon dioxide in the nitrogen after passage through the apparatus was demonstrated qualitatively. At the end of the reaction period benzaldehyde was removed from the reaction mixture by steam distillation and isolated as the phenylhydrazone. The benzaldehyde phenylhydrazone after recrystallization from 60 per cent ethanol had a melting point and mixed<sup>2</sup> melting point of 154–155° with decomposition. The yield was 1 gm; the reaction must have proceeded to at least 25 per cent of completion.

After removal of the benzaldehyde, unchanged aminophenylacetic acid was removed by filtration of the chilled solution. The residue left, upon evaporation of the filtrate to dryness under reduced pressure on a warm water bath, was extracted first with warm acetone to remove unchanged pyruvylalanine and then with 40 cc. of cold water in five portions. The aqueous extract was made just alkaline to litmus by addition of 4 N sodium hydroxide solution and treated with 3 cc. of benzyl chlorocarbonate in 1 cc. portions. During the addition of the chlorocarbonate the reaction

<sup>2</sup> Mixed melting points were always determined simultaneously by placing the substance to be identified, the authentic sample, and their mixture in the same bath.

mixture was shaken thoroughly, cooled, and kept alkaline by the occasional addition of 4 N sodium hydroxide solution. On completion of the reaction, the mixture was shaken with a little charcoal, filtered, and the clear filtrate made distinctly acid to Congo red by the careful addition of concentrated hydrochloric acid. The product separated as an oil which solidified completely after several hours of chilling and scratching. The yield of crude product was 1.9 gm.

To remove any carbobenzoxy-*dl*-alanine which might be present, the crude product was dissolved in 20 cc. of boiling benzene. When the solution was cooled, 1.3 gm. of crystalline material melting over a wide range were deposited. When this material was recrystallized from 5 cc. of ethyl acetate, about 200 mg. of well crystallized material, m.p. 153–156°, separated slowly. After seven recrystallizations from water 53 mg. of this material were recovered in the form of well formed, coarse needles, m.p. 167–167.5°, showing no depression when mixed with Fraction B.

*Analysis*— $C_{14}H_{15}N_2O_4$ . Calculated, N 9.5; found, N 9.5

Careful fractional crystallization of the remaining material from ethyl acetate, ethyl acetate-ligroin mixtures, water, and toluene led only to the isolation of about 100 mg. of carbobenzoxy-*dl*-alanine, m.p. 114–115°, and a small amount (40 mg.) of a carbobenzoxyalanylalanine fraction, m.p. 133–135°, showing no depression when mixed with Fraction C above.

*Analysis*— $C_{14}H_{15}N_2O_5$ . Calculated, N 9.5; found, N 9.6

#### SUMMARY

1. *dl*-Alanylalanine has been synthesized from pyruvylalanine by transamination in a model system with  $\alpha$ -aminophenylacetic acid acting as the amino group donor. The product was isolated as the carbobenzoxy derivative.

2. The preparation of the diastereoisomeric racemates of carbobenzoxy-*dl*-alanyl-*dl*-alanine from alanine anhydride is described.

3. A scheme for the biological synthesis of peptide chains from non-amino acid precursors involving two simple reactions, amination and acylation, is suggested.

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# THE METABOLISM OF *d*-LYSINE INVESTIGATED WITH DEUTERIUM AND HEAVY NITROGEN\*

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It has been recognized for some time that the ability of young rats to utilize for growth certain stereochemically unnatural amino acids must be associated with physiological conversion to the natural configuration (see the discussion in (1, 2)). The mechanism by which this is accomplished *in vivo* has been demonstrated directly in full grown animals. In one case, when *d*- $\alpha$ -amino- $\gamma$ -phenylbutyric acid containing marked nitrogen in the amino group was fed to rats, it resulted in the excretion of the acetylated natural form in which the marked nitrogen had been replaced by normal nitrogen (1). In another case, when *d*-leucine containing  $N^{15}$  in the amino group and deuterium attached to the carbon chain was administered, the natural leucine isolated from the tissue proteins contained deuterium but practically no  $N^{15}$ . During the process of inversion, deamination and reamination had occurred, resulting in replacement of the original nitrogen (2).

The reversible deamination of both natural and unnatural amino acids is now regarded as a rapid and general process in the intact organism, but little is known of the behavior of *d*-lysine. The metabolism of the natural form of lysine is apparently unique in character. When *l*-lysine containing deuterium attached to the carbon chain and  $N^{15}$  in the  $\alpha$ -amino group was fed, the ratio of deuterium to  $N^{15}$  in the lysine isolated from the body proteins was the same as that in the supplement (7). It was evident that the dietary lysine deposited in protein had not undergone reversible deamination. However, the presence of  $N^{15}$  in other amino acids of the tissues and in the excreta indicated that a considerable fraction of the isotopic lysine had been degraded, and it was suggested as one of several possibilities that the absence of nitrogen replacement in the  $\alpha$ -amino group might be due to failure of the keto acid to be reaminated, once this nitrogen has been removed. The failure of  $\alpha$ -hydroxy- $\epsilon$ -amino-*n*-caproic acid to support the growth of immature rats on a lysine-free diet (3) and the observation (4) that a keto acid is excreted by rats when *l*-lysine is added to a protein-free diet suggest a similar possibility.

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† Died, September 11, 1941.

Recognition of a separate amino oxidase system which attacks only the unnatural amino acids makes it apparent that the metabolic behavior of one form of an amino acid cannot be predicted from that of its antipode. *L*-Glutamic acid is reversibly deaminated with great speed, both *in vivo*<sup>1</sup> and *in vitro* (5); yet a large fraction of the unnatural form is found in the urine unchanged with respect to original nitrogen (6). On the other hand, both *d*- and *l*-leucine and *d*- and *l*-tyrosine, when fed in small amounts, are deaminated with equal ease.

The resolution by Weissman and Schoenheimer (7) of isotopic *dl*-lysine containing deuterium attached to the carbon chain and N<sup>15</sup> in the  $\alpha$ -amino group provided the unnatural isomer employed for the present investigation. When administered to growing rats<sup>2</sup> as a dietary supplement in small amounts under the same conditions as those previously employed, *d*-lysine containing the original concentrations of deuterium and N<sup>15</sup> was found in the urine.

*Distribution of N<sup>15</sup> in Urinary Nitrogen*—When the metabolic course of dietary nitrogen is followed by the administration of isotopic amino acids such as glycine (8), *l*-leucine (9), *d*-leucine (2), *dl*-tyrosine (10), or *l*-lysine (7) containing N<sup>15</sup> in the  $\alpha$ -amino group, part of the marked nitrogen was found in the urine. In all these experiments the concentration of N<sup>15</sup> in the total urinary nitrogen was about the same as that in the urea. This was to be expected, since urea normally comprises 80 to 90 per cent of the total urinary nitrogen. The N<sup>15</sup> concentration of other urinary constituents such as ammonia and creatinine, though not identical with that of urea, hardly altered the average value of excreted nitrogen which the N<sup>15</sup> concentration of the total urinary nitrogen represents. As they were present in small amounts, their content of both normal and isotopic nitrogen constituted only a small fraction of the total. The data are given in Table I for comparison with results obtained on feeding isotopic *d*-lysine. In the latter case, the N<sup>15</sup> concentration in the total nitrogen is much higher than in the urea, indicating that a fraction of nitrogen, of very much higher isotope concentration, has been excreted in a form other than urea. It was suspected that the isotope might be located in lysine excreted unchanged in the urine. This was found to be the case.

*Isolation of d-Lysine from Urine*—Lysine was isolated from the urine by precipitation with phosphotungstic acid and purified by recrystallization of the dihydrochloride. A fraction of the isolated material was converted to the dibenzoyl derivative for deuterium analysis. This material proved to be the unnatural form containing the same concentration

<sup>1</sup> Unpublished data.

<sup>2</sup> As in the experiment with *l*-lysine, young animals were employed in order to obtain conditions of maximum amino acid demand.

of  $N^{15}$  and deuterium as the compound which was fed. There can be little doubt that the amino acid was excreted as such, as the isolation procedure was unlikely to hydrolyze any but the most labile linkage.

*Lysine in Tissue Proteins*—The *d*-lysine employed for feeding was synthesized so as to contain deuterium stably bound in the  $\beta$ ,  $\gamma$ , and  $\delta$  positions. If after ingestion reversible deamination and inversion had occurred, permitting utilization of the original carbon chain for amino acid synthesis, deuterium, but not  $N^{15}$ , would be present in the lysine of the body proteins. However, the lysine isolated from the rat tissues was found to be of natural configuration and contained neither deuterium nor  $N^{15}$ . Failure to utilize the carbon chain of ingested *d*-lysine is in entire agreement with the results obtained after the natural form is fed, for

TABLE I

*Concentration of  $N^{15}$  in Total Nitrogen and Urea of Urine after Feeding Isotopic Amino Acids*

The values are calculated on the basis of 100 atom per cent  $N^{15}$  in the compound administered.

Amino acid administered	$N^{15}$ concentration in urea nitrogen	$N^{15}$ concentration in total urinary nitrogen
	atom per cent	atom per cent
Glycine.....	2.7	2.8
<i>l</i> -Leucine.....	2.4	2.3
<i>d</i> -Leucine.....	4.3	4.8
<i>dl</i> -Tyrosine.....	3.4	3.4
<i>l</i> -Lysine.....	2.4	2.1
<i>d</i> -Lysine.....	1.0	5.8

unless reamination of the intermediate keto acid can take place, inversion does not occur.

*Distribution of Ingested Lysine Nitrogen*—Most of the  $N^{15}$  (69 per cent) was found in the urine (Table II). Though an appreciable fraction of the ingested *d*-lysine was excreted, some degradation had occurred, resulting in the utilization of ingested isotopic nitrogen. 21 per cent of the administered isotope was found in the tissue proteins and was present in amino acids other than lysine (Table III). Only 5.5 per cent was present in the non-protein nitrogen fraction. The fraction found in the feces was small (1.5 per cent), indicating that the material was well absorbed.

A rough estimate of the amount of unchanged *d*-lysine excreted may be made on the assumption that of the total urinary nitrogen, approximately 80 per cent is urea and 10 per cent is ammonia. These urinary constituents, which contained 0.046 and 0.189 atom per cent  $N^{15}$  respectively, account for 19 per cent of the  $N^{15}$  added to the diet. The remaining  $N^{15}$

in the urine (50 per cent of the total fed) probably exists mainly as *d*-lysine, of which 895 mg. should be present. Approximately one-third of this amount was recovered in pure form. No attempt was made to obtain a high yield during the isolation procedure; the aim was to secure material of sufficiently high purity for isotope analysis.

TABLE II

*Balance of Nitrogen Isotope after Feeding Isotopic d(-)-Lysine*

The values are calculated from the total nitrogen of the fractions and their isotope concentrations.

	Fraction of administered N <sup>15</sup> recovered
	<i>per cent</i>
Feces.....	1.5
Urine.....	69.1
Non-protein nitrogen .....	5.5
Protein nitrogen .....	21.4
Total isotope recovered... ..	97.5

TABLE III

*N<sup>15</sup> Concentration in Protein Constituents from Rats Given Isotopic d(-)-Lysine*

The values are calculated for an isotope concentration of 100 atom per cent in the  $\alpha$ -amino group of the administered compound. The error of these values is  $\pm 0.07$  per cent.

Compound isolated	N <sup>15</sup> concentration	Compound isolated	N <sup>15</sup> concentration
	<i>atom per cent</i>		<i>atom per cent</i>
Total protein .....	0.31	Aspartic acid .....	0.31
Amide N .....	0.59	Glutamic " .....	0.22
Arginine.....	0.24	Lysine .....	0.09

## EXPERIMENTAL

Details of the preparation of the isotopic *d*-lysine have been published (7). The dihydrochloride contained 6.9 atom per cent deuterium (corresponding to 7.9 atom per cent in the lysine itself) and 2.2 atom per cent N<sup>15</sup> excess. As the N<sup>15</sup> was present in the  $\alpha$ -amino group only, this group had an isotope concentration of 4.4 atom per cent N<sup>15</sup> excess. It contained 12.6 per cent N by Kjeldahl analysis (theory for C<sub>6</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>Cl<sub>2</sub>, calculated for 7 atom per cent deuterium, 12.7 per cent).

$$[\alpha]_D^{25} = -15.7^\circ \text{ (2.9\% in H}_2\text{O)}$$

*Feeding Experiment*—Three young male rats having a combined weight of 311 gm. consumed 135 gm. of stock diet containing 1.79 gm. of isotopic lysine dihydrochloride over a 4 day period. The latter was added to the daily food allotment as a solution along with 2 equivalents of sodium hydroxide. An amount of sodium chloride equal to that supplied in the supplement was omitted from the usual salt mixture. Urine and feces were collected quantitatively in metabolism cages. At the end of the experimental period the combined weight of the animals was 354 gm.

*Analysis of Excreta*—The combined feces were dissolved by digestion in hot concentrated  $\text{H}_2\text{SO}_4$  and aliquots employed for analysis. The feces contained 330 mg. of N having 0.024 atom per cent  $\text{N}^{15}$  excess. The pooled urine of the first 3 days contained 1.428 gm. of nitrogen having 0.216 atom per cent  $\text{N}^{15}$  excess. The last day's urine, which was treated separately, contained 169 mg. of nitrogen with 0.256 atom per cent  $\text{N}^{15}$  excess. Urea, isolated as the dioxanthryl derivative, had 0.046 atom per cent  $\text{N}^{15}$  and 6.7 per cent N (theory 6.7 per cent N). A sample of ammonia was obtained by treatment with permittit and subsequent distillation. It had 0.189 atom per cent  $\text{N}^{15}$ .

*Isolation of d-Lysine from Urine*—After the removal of samples for analysis, the urine of the entire period was combined, concentrated *in vacuo* to 35 cc., and treated with mercuric acetate and sodium carbonate, according to the directions of Neuberg and Kerb (11). Mercury was removed from the insoluble fraction by treatment with  $\text{H}_2\text{S}$  and the lysine precipitated by phosphotungstic acid. This precipitate was decomposed by extracting the suspension in dilute HCl with butyl alcohol-ether mixture in the usual manner. The aqueous layer was concentrated to a syrup and taken up in 10 cc. of alcohol. Insoluble material was removed by filtration and 30 cc. of acetone were slowly added to the filtrate. The crystalline mass which formed on standing was recrystallized three times in a similar manner by dissolving in a few drops of water, adding 5 cc. of alcohol, and finally 15 to 20 cc. of acetone. The resulting 289 mg. of purified lysine dihydrochloride contained 12.6 per cent N (Kjeldahl) and 2.23 atom per cent  $\text{N}^{15}$ .

$$[\alpha]_D^{25} = -15.9^\circ \text{ (2.2\% in H}_2\text{O)}$$

Part of this material was converted to dibenzoyllysine, which, after recrystallization once from water and twice from acetone, melted at 149–150°. It contained 7.75 per cent nitrogen by Kjeldahl analysis (theory 7.9 per cent) and 5.08 atom per cent deuterium which corresponds to 7.00 atom per cent deuterium for lysine dihydrochloride.

*Isolation of Amino Acids from Body Proteins*—The intestinal contents were removed, and the whole animals were minced and extracted with 6

per cent trichloroacetic acid in the usual manner. The extract, constituting the non-protein nitrogen fraction, contained 1.025 gm. of nitrogen and 0.027 atom per cent  $N^{15}$  excess. The protein residue was hydrolyzed in boiling 20 per cent  $H_2SO_4$  for 24 hours and the acid removed by neutralization with  $Ba(OH)_2$ . The hydrolysate contained 7.69 gm. of nitrogen and 0.014 atom per cent  $N^{15}$  excess. 0.23 gm. of amide nitrogen containing 0.027 atom per cent  $N^{15}$  was removed by distillation *in vacuo* in the presence of excess  $Ba(OH)_2$ . Glutamic and aspartic acids, precipitated as the barium salts, were isolated as copper aspartate containing 7.10 per cent N (Kjeldahl) and 0.014 atom per cent  $N^{15}$  excess and glutamic hydrochloride containing 7.60 per cent N and 0.010 atom per cent  $N^{15}$  excess. Arginine was isolated as the flavianate and converted to the *p*-toluenesulfonyl derivative. This melted at 260–261° with decomposition; it contained 14.8 per cent N (Kjeldahl) (theory 14.7 for  $C_{13}H_{20}O_4N_4S \cdot 3H_2O$ ) and 0.011 atom per cent  $N^{15}$ .

Lysine and histidine were precipitated by means of phosphotungstic acid and the precipitate decomposed. Histidine was precipitated with  $HgCl_2$  at pH 7 (12); lysine was isolated from the supernatant as the dihydrochloride and purified as described above. 425 mg. were finally obtained.

$$[\alpha]_D^{25} = +15.8^\circ \text{ (2.97\% in } H_2O \text{)}$$

N 12.7 per cent (Kjeldahl) contained 0.004 atom per cent  $N^{15}$ .

Part of the material was converted to the dibenzoyl derivative for deuterium analysis. It contained 7.82 per cent N (Kjeldahl) and 0.02 atom per cent deuterium.

#### DISCUSSION

Although the excretion of *D*-lysine has not been reported previously, the appearance of other unnatural amino acids in the excreta of normal animals is well known. Wohlgemuth (13) found in 1905 that rabbits excreted *D*-tyrosine, *D*-glutamic acid, *D*-leucine, and *D*-aspartic acid when these amino acids were given in racemic form. Both the dog (14) and the cat (15) have been found to behave similarly with respect to tyrosine. However, it appears from the data in Table I that the rat can deaminate both *D*-tyrosine and *D*-leucine adequately when these amino acids are offered in moderate amounts. Similar results were also obtained with *D*-aspartic acid.<sup>1</sup> Apparently excretion of these three amino acids occurs only when the existing mechanism is overwhelmed by an excessive supply. The ability of the rat to deaminate *D*-lysine appears to be more restricted,<sup>3</sup>

<sup>3</sup> *D*-Glutamic acid was also found to be largely excreted (6) and should be classified with lysine in this respect.

for when given in approximately the same moderate amount as the other isotopic amino acids (19 mg. of lysine nitrogen per rat per day, corresponding to 6 per cent of the dietary nitrogen) at least half of the original material was excreted. It is probably pertinent that *d*-lysine has been found to resist the action of *d*-amino oxidase (16, 17).

Since in the present experiments  $N^{15}$  was found in urea and ammonia and in the proteins of the tissues, some degradation of dietary *d*-lysine must have occurred, but the deaminative activity of the liver and kidney toward *d*-lysine can be only slight. Transfer of nitrogen via transamination is hardly likely, since this enzyme system exhibits only negligible activity toward the unnatural amino acids (18).

In any case, the present experiments show that the extent to which *d*-lysine is broken down by the rat is highly limited.

It is to be expected, in general, that the rate of deamination of the unnatural amino acids will affect their availability for growth or maintenance. Although most of the essential amino acids can support the growth of rats when supplied in the unnatural form, *d*-leucine (19) and *d*-lysine (3, 20) are exceptions. The respective reasons for this exceptional behavior, however, are different. The deamination and inversion of *d*-leucine has been found to occur in adult rats (2) but the rate of this process is evidently insufficient to supply the whole growth requirement. On the other hand, the limiting factor in the case of *d*-lysine appears to be the non-reamination of the keto acid rather than its slow rate of appearance; the carbon chain is completely unavailable.

#### SUMMARY

1. Isotopic *d*(-)-lysine containing heavy nitrogen in the  $\alpha$ -amino group and deuterium attached to the carbon chain was administered for 4 days in small amounts to young rats by addition to the stock diet.

2. Approximately half of the ingested amino acid was excreted in the urine. Optical rotation and isotope analyses of the isolated material have shown it to be identical with the ingested compound.

3. A fraction of the administered *d*(-)-lysine was broken down with loss of the  $\alpha$ -amino group. This marked nitrogen appeared partly (19 per cent) in the urine, as ammonia and urea, and partly (21 per cent) in various amino acids of the tissue proteins.

4. *l*(+)-Lysine isolated from the tissue proteins contained neither deuterium nor heavy nitrogen, indicating that the carbon chain of *d*(-)-lysine is unavailable for protein synthesis. The inability of the rat to convert *d*-lysine to lysine having the natural configuration is in harmony with the previous observation that *l*-lysine is not regenerated *in vivo* by reamination of its breakdown products.



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## A COLOR REACTION FOR METHIONINE

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Because of the increasing research on the rôle of the amino acids, their preparation in pure form is of primary importance. In establishing the purity of a compound, it is often desired to determine the absence of a specific contaminant. Methionine, an impurity likely to be present in commercial *L*-leucine derived from natural sources, has been found to give a yellow color with a saturated solution of anhydrous cupric sulfate in concentrated sulfuric acid. This color reaction resembles that previously reported by Kolb and Toennies,<sup>1</sup> who obtained a pale yellow to deep brown color, according to the concentration of methionine, with a solution of cupric chloride in concentrated hydrochloric acid. Because of the interfering color of the cupric chloride-hydrochloric acid reagent, the sensitivity of their test is relatively low. Kolb and Toennies state that cupric chloride solutions in concentrated sulfuric, perchloric, or acetic acid do not produce a color with methionine.

**Cupric Sulfate-Sulfuric Acid Reagent**—The reagent may be prepared by warming a slight excess of anhydrous cupric sulfate with concentrated sulfuric acid, cooling to room temperature, and allowing to settle. The clear, colorless, supernatant liquid is used.

One batch of the reagent made from sulfuric acid containing 96.3 per cent  $\text{H}_2\text{SO}_4$  and from anhydrous copper sulfate (98.1 per cent  $\text{CuSO}_4$  on analysis) was found to contain 1.1 mg. of  $\text{CuSO}_4$  per ml. at 25°. A second batch prepared from the same anhydrous copper sulfate but with other sulfuric acid, which assayed 95.8 per cent  $\text{H}_2\text{SO}_4$ , contained 1.3 mg. of  $\text{CuSO}_4$  per ml.

**Sensitivity**—It was found that the minimal concentration of methionine which can be detected visually with certainty is 0.1 mg. per ml. of reagent. In a volume of 1.0 ml. of the reagent increments of 0.1 mg. can be differentiated up to the level of 1.0 mg. of methionine. In the range of from 1.0 to 2.0 mg., it is possible to distinguish increments of 0.2 mg., and as the methionine concentration increases further, the increments that can be differentiated become greater.

**Procedure for Detecting and Estimating Methionine in *L*-Leucine**—Place 100 mg. of the sample of *L*-leucine in a small test-tube (3 inch) and add 1.0 ml. of the reagent. Shake or mix with a small stirring rod to facilitate

<sup>1</sup> Kolb, J. J., and Toennies, G., *J. Biol. Chem.*, **131**, 401 (1939).

solution. If the resulting solution shows no yellow color when compared with a blank, it may be concluded that less than 0.1 mg. of methionine is present or less than 0.1 per cent on a percentage basis. The formation of a yellow color, however, indicates the presence of methionine.

It was found by preparing a series of standards with and without leucine that the yellow color produced by methionine is intensified in the presence of leucine. The color of 0.2 mg. of methionine plus 100 mg. of leucine in 1 ml. of the reagent matches that of 0.3 mg. of methionine in 1 ml. of the reagent without any leucine. 0.6 mg. of methionine plus 100 mg. of leucine matches approximately 1.1 mg. of methionine without leucine; 1.2 mg. of methionine plus 100 mg. of leucine, about 2.5 mg. of methionine without leucine; and 1.8 mg. of methionine plus 100 mg. of leucine, about 3.3 mg. of methionine without leucine. In compensation for the intensification of color due to leucine, standards prepared for matching the yellow color must contain a quantity of methionine-free leucine equal to the

TABLE I  
*Determination of Methionine Content of Unknowns*

Unknown No.	Methionine content	Found by	
		Chemist A	Chemist B
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.2	0.25	0.2
2	0.8	0.8	0.8
3	1.6	1.5	1.5

weight of the sample taken. Synthetic *dl*-leucine, previously shown to dissolve in the reagent without any color, may be used for this purpose.

Using a standard solution of methionine in the reagent containing 2.0 mg. of methionine per ml., prepare the necessary standards for matching the color produced by the sample. Each standard contains 100 mg. of *dl*-leucine (or methionine-free *l*-leucine), and a known volume of the standard methionine solution, plus a volume of the reagent to make the final volume 1.0 ml. By spacing the standards at intervals of 0.1 ml. of the standard solution (0.2 mg. of methionine), the methionine content of the sample of *l*-leucine can be estimated to within 0.1 mg. or within 0.1 per cent on the basis of a 100 mg. sample. If the methionine content of the *l*-leucine is more than 2.0 per cent, a smaller sample, *e.g.* 50 mg., should be used with a corresponding reduction in the amount of *dl*-leucine in the standards. If the methionine content is less than 1.0 per cent, it can be determined within 0.05 per cent by the use of a standard solution containing 1.0 mg. of methionine per ml., the standards being prepared at increments of 0.1 mg.

To illustrate the results that can be obtained, we list (Table I) the values obtained by two chemists on three unknowns prepared from synthetic *dl*-leucine and *dl*-methionine.

*Selectivity*—The reagent reacts with halides and, therefore, we converted the hydrochlorides of arginine, histidine, and lysine to the sulfates before determining whether or not these amino acids produce any color with the reagent.

The following amino acids were found to give no reaction with the reagent: alanine, arginine (sulfate), aspartic acid, cystine, glutamic acid, glycine, histidine (sulfate), hydroxyproline, isoleucine, leucine, lysine (sulfate), norleucine, phenylalanine, proline, serine, threonine, valine. *Tryptophane* gives a bright yellow color with a slight fluorescence; *tyrosine*, a yellow color of lesser intensity and different shade from that given by methionine. Satisfactory tests are available for the detection of both tryptophane and tyrosine, and their presence or absence in a sample of *l*-leucine to be tested for methionine can be determined. In the purification of *l*-leucine, tryptophane and tyrosine do not present the same difficulty as methionine. The two amino acids containing iodine, *diiodo-tyrosine* and *thyroxine*, decompose when treated with the reagent, but these are not likely contaminants of *l*-leucine because of their limited occurrence.

#### SUMMARY

Methionine gives a yellow color with a saturated solution of anhydrous cupric sulfate in concentrated sulfuric acid. This color reaction can be used for the detection and estimation of methionine in *l*-leucine. 0.1 mg. of methionine can be detected in a volume of 1.0 ml. of the reagent, corresponding to 0.1 per cent methionine on the basis of a sample of 100 mg.



# THE PREPARATION AND COMPARATIVE PHYSIOLOGICAL ACTIVITIES OF BEEF, HOG, AND SHEEP ADRENAL CORTEX EXTRACTS

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Our knowledge of the chemical and physiological properties of compounds occurring in the adrenal cortex is based on investigations conducted almost exclusively with extracts and isolation products of beef adrenals. More than twenty-five pure steroids have been isolated but only a relatively few have been found to be biologically active. The amounts and proportions of active steroids present in the adrenals of different species have not been thoroughly determined. We are at present engaged in the fractionation of adrenal cortex extracts prepared from beef, hog, and sheep glands. This report is concerned with the preparation of extracts from each of these species and with a comparative study of their biological activities.

The preparations were assayed in adrenalectomized rats by the following two methods: (a) The survival-growth test, as previously reported from this laboratory (1), is a useful method for determining the complete cortical hormone activity of the whole extracts. All of the known steroids which have the property of replacing, at least in part, the functional activity of the adrenal cortex have been found active by this test. (b) The work performance test in adrenalectomized rats is believed to be specific for the 11-oxygenated sterols which affect carbohydrate metabolism. The previously described method (2, 3) has been modified for the purpose of quantitative assay. A detailed description of the apparatus and procedures will be published in a separate paper. 1 unit of activity is defined as the work equivalent of 0.2 mg. of 17-hydroxy-11-dehydrocorticosterone.

## EXPERIMENTAL

The hog, beef, and sheep glands were collected and frozen at the packing house and shipped to the laboratory packed in dry ice. In each case the glands were finely ground while frozen and transferred immediately to acetone and processed as previously described for beef adrenal glands (4). For the beef and sheep glands, this procedure, which consists of acetone extraction for 5 days, filtration from gland residue, evaporation of acetone, extraction of the aqueous solution with petroleum ether of boiling point 60-70°, followed by extraction with ethylene dichloride, yields an extract

containing between 35 and 45 gm. of solids per 1000 pounds of gland processed.

In the attempt to work up hog adrenals by this procedure, considerable difficulty was encountered because of the exceedingly high content of fat. Before concentration of the acetone extract, it was necessary to dilute with sufficient water to make a 50 per cent acetone solution. A liquid fat fraction separated from this aqueous acetone which was separated by decantation. This fat was reextracted with 40 per cent acetone and the combined aqueous acetone solutions were concentrated *in vacuo* to remove acetone. The aqueous solution was then extracted with petroleum ether, followed by extraction with ethylene dichloride to obtain the active fraction. The ethylene dichloride-soluble material was transferred to 95 per cent alcohol and more fat and cholesterol were removed by extraction of aqueous alcohol solutions with petroleum ether, which was done first from 70 per cent ethyl alcohol, then from 50 per cent methyl alcohol, and finally from 30 per cent methyl alcohol. In the case of the beef extract the distribution between 30 per cent methanol and petroleum ether resulted in all the material remaining in the 30 per cent methanol layer. The hog and sheep extracts, however, were not completely free of fat until distribution was carried out between 30 per cent methanol and petroleum ether. The dilute methanol solutions free of petroleum ether-soluble material were concentrated to remove alcohol and the cloudy aqueous solutions were extracted with ethyl acetate. Acidic and basic substances were removed by washing the ethyl acetate solution with sodium carbonate followed by 0.5 N HCl and distilled water as earlier described (5).

### Results

The entire fat fraction from hog gland amounted to about 175 pounds per 1000 pounds of gland, whereas the beef and sheep fat fractions weighed only 20 pounds per 1000 pounds of gland processed. The ethylene dichloride extract of hog gland also contained 10 times as much gland extractives as either the sheep or beef extract.

The results of the fractionation with particular reference to the amount of solids at the various steps, beginning with the ethylene dichloride solution, are summarized in Table I.

In Table II the comparative biological activities of the different fractions by the two assay methods are recorded. In order to present data on the recovery of activity after removal of acidic and basic material, assays of the 30 per cent methanol solutions are also given. The biological activity is given in units by the muscle contraction test of Ingle, in which 1 unit is equivalent to 0.2 mg. of 17-hydroxy-11-dehydrocorticosterone, and in units by the survival test, in which the unit, as previously defined

(1), is the minimum amount of hormone necessary to maintain 80 per cent of adrenalectomized 4 week-old rats for an injection period of 20 days and produce an average growth per rat of at least 20 gm. over this period. All assay samples were made up in sesame oil and the volume of oil injected was between 0.1 and 0.2 cc. per day in the survival test and 1.0 cc. in the muscle contraction test.

As can be seen from Table II, the biological activity of the hog extract was considerably higher than that of the beef or sheep extracts. By the survival test in adrenalectomized rats the hog extract was 50 per cent more active than the beef extract and 100 per cent more active than the sheep extract. By the muscle contraction test it was found 64 per cent more potent than the beef and 89 per cent more potent than the sheep extracts. Vars, Taylor, and Piffner (6), comparing the yields of "cortical hormone" from 5 pound quantities of gland by the dog assay method (7), reported that both hog and sheep extracts were about 40 per cent more active than

TABLE I

*Comparison of Extracts from 900 Kilos of Beef, Hog, and Sheep Adrenal Glands*

Fraction	Beef	Hog	Sheep
	gm.	gm.	gm.
Ethylene dichloride.....	70	900	75
70% ethanol.....	34	212	50
50% methanol.....	25	49	41
30% ".....		39	31
Neutral ethyl acetate.....	18.73	25.88	14.82

beef extracts. Our results by the two different assay methods substantially confirm the findings of Vars *et al.* with respect to the hog extract, but not with respect to the sheep extract; the latter was found by us to be somewhat less active than the beef extract.

Complete recovery of activity, after removal of acidic and basic material, was found by both assay methods. There was indeed a slight increase by the muscle contraction test, which was greater for the sheep extract than for the hog. We attributed this increase, if significant at all, to the removal of toxic constituents.

The results by the muscle contraction test indicate that the high activity of the hog extract is due to its high content of those adrenal steroids which bear an oxygen atom at carbon atom 11, and which are active in carbohydrate metabolism. The increased activity of the hog extracts by the growth-survival test may therefore also be due to the higher concentration of these compounds.



TABLE II  
Comparison of Biological Activity from Beef, Hog, and Sheep Adrenal Extracts

900 kilos of glands were extracted in each case.

Fraction	Beef				Hog				Sheep						
	Weight		Biological activity		Weight		Biological activity		Weight		Biological activity				
			Muscle test	Survival test			Muscle test	Survival test			Muscle test	Survival test			
	gm.	units per mg.	total units	units per mg.	total units	gm.	units per mg.	total units	gm.	units per mg.	total units	units per mg.	total units		
30% methyl al- cohol.....	25.96	0.94	24,400	5.2	135,000	39.0	1.03	40,000	5.77	225,000*	31.0	0.55	17,050	3.63	112,500†
Neutral ethyl acetate.....	18.73	1.37	25,700	8.00	150,000	25.88	1.64	42,443	8.69	225,000*	14.82	1.51	22,378	7.59	112,500†

\* The 30 per cent methyl alcohol fraction and the neutral ethyl acetate fraction were assayed at levels of 12, 8, 6, 4, 3, and 2 gm. equivalents of gland per rat per day. In both fractions there was found to be 1 unit per 4 gm. of gland, since all rats receiving more than this were well maintained and all receiving less were not maintained.

† The sheep extracts were assayed at levels of 12, 8, 6, and 4 gm. equivalents of gland per rat per day. The rats failed at levels of 6 and 4 but were maintained at 12 and 8 gm. equivalents; so that there is 1 unit per 8 gm. of gland.

## SUMMARY

Beef and sheep adrenal cortex extracts were prepared by the standard procedure previously reported (4). Because of the high content of fat in hog adrenal glands, this procedure was modified slightly for processing these glands. A comparison of the biological activities of these extracts was made by both the rat survival test and by the Ingle muscle contraction test. It was found that the hog adrenal extract was considerably more active by both tests than either beef or sheep adrenal extracts, and it was concluded that this higher activity may be due to increased amounts of 11-oxygenated sterols in the hog extract.

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## THE ACONITE ALKALOIDS

### XI. THE ACTION OF METHYL ALCOHOLIC SODIUM HYDROXIDE ON ATISINE. ISOATISINE AND DIHYDROATISINE

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(Received for publication, December 28, 1942)

We have recently described<sup>1</sup> the production from the alkaloid atisine of a possible dihydroatisine under the influence of saturated methyl alcoholic NaOH solution at 100°. Because of the unusual nature of the apparent disproportionation of hydrogen which could lead to such a result, we have studied the subject further. The more recent results appear to have confirmed our original observations and conclusions, and to have demonstrated the production of dihydroatisine in a yield of at least 75 per cent when atisine is heated for 90 hours with the above reagent. The analysis of the hydrochloride prepared from this supported the formulation  $C_{22}H_{33}O_2N \cdot HCl$ . As additional substantiation, we have again found that the base, as well as its hydrochloride, absorbs only 1 mole of  $H_2$  to produce a mixture of isomers from which the tetrahydroatisine melting at 172–173° could be fractionated. This substance agreed in all properties with the tetrahydroatisine obtained from atisine itself by the absorption of 2 moles of  $H_2$ .

If, however, atisine is subjected to gentler treatment with alkali, it has now been found that its transformation can be intercepted at the stage of a beautifully crystalline *isoatisine*,  $C_{22}H_{33}O_2N$ , which melts at 150–151° ( $[\alpha]_D^{25} = -16.5^\circ$  ( $c = 1.15$  in toluene)). This conversion is almost quantitative. *Isoatisine hydrochloride* ( $[\alpha]_D^{25} = -4^\circ$  ( $c = 0.98$  in  $H_2O$ )) melts with decomposition at 295–299°. *Isoatisine*, either as the base or hydrochloride, absorbs 2 moles of  $H_2$  on hydrogenation to form a mixture from which the above tetrahydroatisine can be obtained.

#### EXPERIMENTAL

*Dihydroatisine*—1.2 gm. of atisine hydrochloride were sealed in a tube with 22 cc. of methanol saturated by heating with NaOH. The mixture which congealed at room temperature to a pap of sodium methylate was heated at 100° for 90 hours. The diluted mixture was extracted with ether, and this after drying and concentration yielded 0.62 gm. of crystalline base which softened and gradually melted at 144–149°. The mother liquor yielded 0.2 gm. of a second crop of crystals or in all 75 per cent of

<sup>1</sup> Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **143**, 589 (1942).

the theory. The first crop was dissolved in 6 cc. of 95 per cent alcohol, gently warmed, and treated with 4 cc. of water. On seeding, a mass of small glistening prisms and rhombs separated which gradually softened to a paste above  $142^{\circ}$  but was not completely melted until  $149-151^{\circ}$ .

$$[\alpha]_D^{25} = -44^{\circ} (c = 1.05 \text{ in toluene})$$

$C_{22}H_{35}O_2N$ . Calculated, C 76.46, H 10.22; found, C 76.54, H 10.40

The hydrochloride was prepared from the base by solution in 95 per cent alcohol containing an excess of HCl. On addition of ether, it crystallized readily as needles which melted with decomposition at  $259^{\circ}$  (uncorrected) after preliminary sintering.

$C_{22}H_{35}O_2N \cdot HCl$ . Calculated. C 69.16, H 9.50, Cl 9.29  
Found. " 69.15, " 9.63, " 9.32

We have previously recorded the behavior of dihydroatisine both as the base and the hydrochloride on hydrogenation. This experience was confirmed with the above dihydroatisine.

0.2 gm. of this material, when hydrogenated in methanol with 50 mg. of platinum oxide catalyst, absorbed 12 cc. (or about 1 mole) in excess of the catalyst. On recrystallization from methanol, 28 mg. of needles were obtained which melted at  $172-173^{\circ}$  (uncorrected).

$$[\alpha]_D^{25} = -10^{\circ} (c = 1.03 \text{ in pyridine})$$

$C_{22}H_{37}O_2N$ . Calculated, C 76.02, H 10.74; found, C 75.94, H 10.65

Tetrahydroatisine obtained from atisine hydrochloride itself by the absorption of 2 moles of  $H_2$  melted at  $172-173^{\circ}$  and gave  $[\alpha]_D^{27} = -11^{\circ} (c = 0.91 \text{ in pyridine})$ .

*Isoatisine*—5 gm. of atisine hydrochloride were dissolved in a solution of 7.5 gm. of NaOH in 125 cc. of methanol. After 11 days, the colorless solution was diluted and extracted with chloroform. The latter yielded a residue which readily crystallized from dilute alcohol as small, glistening, four-sided, or rhombic prisms, which melted at  $150-151^{\circ}$  after preliminary softening. The yield was 86 per cent of the theory.

$$[\alpha]_D^{25} = -16.5^{\circ} (c = 1.15 \text{ in toluene})$$

$C_{22}H_{33}O_2N$ . Calculated. C 76.91, H 9.69, N 4.08  
Found. " 76.85, " 9.43, " 4.09

The hydrochloride separated from alcohol-ether as minute, lustrous, six-sided platelets, which gradually melted with decomposition at  $295-299^{\circ}$  after preliminary sintering.

$$[\alpha]_D^{25} = -4^{\circ} (c = 0.98 \text{ in } H_2O)$$

$C_{22}H_{33}O_2N \cdot HCl$ . Calculated, C 69.52, H 9.02; found, C 69.49, H 8.86

An almost quantitative conversion of atisine into isoatisine was accomplished by refluxing for  $3\frac{1}{2}$  hours 1 gm. of the hydrochloride in a solution of 3 gm. of NaOH in 40 cc. of methanol.

On hydrogenation, isoatisine, both as the base or as the hydrochloride, was found to yield a mixture of tetrahydroatisines as follows:

0.2 gm. of isoatisine absorbed 2 moles of  $H_2$  on hydrogenation in methanol solution with platinum oxide catalyst. The resulting mixture on fractionation yielded 43 mg. of tetrahydroatisine, which melted at  $172-174^\circ$ .

$C_{22}H_{17}O_2N$ . Calculated, C 76.02, H 10.74; found, C 76.04, H 10.57

0.3 gm. of isoatisine hydrochloride absorbed 2 moles of  $H_2$  in methanol solution with platinum oxide catalyst. After recrystallization of the free base isolated from the reaction mixture, 52 mg. of tetrahydroatisine resulted, which melted at  $172-175^\circ$ .

$$[\alpha]_D^{25} = -10^\circ (c = 1.01 \text{ in pyridine})$$

Found. C 76.05, H 10.65; C 76.14, H 10.85

On further treatment with methyl alcoholic alkali as in the case of atisine itself, isoatisine was, as expected, converted into the above dihydroatisine.

The microanalytical determinations, as in the past, have been made by Mr. D. Rigakos of this laboratory.



# THE ACONITE ALKALOIDS

## XII. BENZOYL HETERATISINE, A NEW ALKALOID FROM ACONITUM HETEROPHYLLUM

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(Received for publication, December 28, 1942)

In a former paper (1) on the procedure employed for the isolation of the alkaloid atisine from atis root, it was mentioned that the dilute  $\text{H}_2\text{SO}_4$  solution of the crude alkaloids was neutralized with  $\text{Na}_2\text{CO}_3$  solution, and a preliminary extraction with benzene was then made before the addition of an excess of  $\text{NaOH}$  solution and final extraction of the atisine fraction itself. A more recent examination of this  $\text{Na}_2\text{CO}_3$  fraction has demonstrated the presence in it of a new alkaloid, *benzoyl heteratisine*,  $\text{C}_{27}\text{H}_{37}\text{O}_6\text{N}$ . For purification, this benzene solution, which had accumulated from the extraction of a large amount of plant material, was exhaustively extracted with 2 per cent  $\text{H}_2\text{SO}_4$ . The latter was neutralized with excess  $\text{Na}_2\text{CO}_3$  solution and again extracted with benzene. This extract yielded on concentration a syrup which gradually crystallized on standing. After digestion with a small volume of benzene, the crystals were collected with this solvent. 200 pounds of the crude drug yielded more than 6 gm. of the new alkaloid.

The latter crystallized from benzene as prisms, which melted at  $213\text{--}214^\circ$  after preliminary softening.

$[\alpha]_D^{25} = +73^\circ$ ( $c = 1.0$ in 95% ethanol)		
$\text{C}_{27}\text{H}_{37}\text{O}_6\text{N}$ .	Calculated.	C 70.26, H 7.53, N 2.83
	Found.	" 70.18, " 7.68, " 3.00

The *hydrochloride* separated from methanol-ether as needles, which gradually melted with decomposition at  $218\text{--}221^\circ$  after preliminary darkening and softening.

$\text{C}_{27}\text{H}_{37}\text{O}_6\text{N} \cdot \text{HCl}$ .	Calculated.	C 65.44, H 7.20, Cl 6.67
	Found.	" 65.54, " 7.47, " 6.37

From the derived formulation and the behavior toward alkali, the new alkaloid was shown to be the benzoyl derivative of the alkaloid, heteratisine, recently isolated by us (2) from the mother liquors of atisine.

For saponification, 0.12 gm. was gently warmed in a mixture of 5 cc. of methanol and 0.5 cc. of 25 per cent  $\text{NaOH}$ . The odor of benzoic ester at once became apparent. After concentration to remove most of the solvent and dilution, the clear solution was acidified to Congo red with



HCl to reacetone the saponified heteratisine. After standing, the solution was treated in the cold with excess alkali, and quickly extracted with chloroform. The latter yielded a residue which crystallized from alcohol in characteristic trapezoidal and rhombic prisms. These melted with slow effervescence at 265–267°, and proved to be heteratisine.

$$[\alpha]_D^{25} = +40^\circ (c = 1.05 \text{ in methanol})$$

$C_{22}H_{33}O_5N$ . Calculated, C 67.47, H 8.50; found, C 67.75, H 8.56

The above aqueous alkaline fraction of the saponification mixture, after acidification and extraction, yielded benzoic acid (m.p. 122–123°).

It is possible that heteratisine does not occur as such in *Aconitum heterophyllum* but is an artefact produced from the benzoyl derivative during the isolation process.

The microanalyses were made by Mr. D. Rigakos.

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# THE PREPARATION OF CONCENTRATES OF SPECIFIC SUBSTANCES FROM URINE AND FECES IN LEUCEMIA

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(Received for publication, December 29, 1942)

Miller, Wearn, and Heinle (1, 2) found that the injection of certain fractions from the urine of patients with myeloid and lymphoid leucemia produced characteristic changes in the organs of guinea pigs. These changes resembled the histological findings in human leucemia and were specifically myeloid or lymphoid, corresponding to the disease in the patients from whom the urine was obtained. Fractions from the urine of patients with other diseases or similar fractions from normal urine did not produce these specific histological pictures. The urine fractions were originally prepared by acid hydrolysis followed by adsorption on kaolin. Later (3), they were made by acid hydrolysis followed by chloroform extraction. Experiments of Miller, Hause, and Jones (4) showed that the active material of myeloid urine accumulated solely in the fraction containing the acids and phenols, and that it was soluble in ether, and petroleum ether.

The present paper reports the further concentration of the active material from the urine of patients with myeloid and lymphoid leucemia. The biological reaction from the more concentrated material is much more pronounced and specific than any which was obtained from cruder fractions. Since the biological test is not quantitative, a qualitative scheme is used to compare fractions. Those fractions which produce specific myeloid or lymphoid proliferation in the organs of guinea pigs are designated as positive (+), and those fractions which do not produce such a proliferation are described as negative (-). By a repetition of plus signs, the intensity of the response is roughly designated. The most important feature of the biological reaction for the chemical work is its myeloid or lymphoid specificity.

The separations shown in the flow sheet have been applied to the urine of patients with myeloid, lymphoid, and monocytic leukemia, and also to the urine of patients with the leukemia-like diseases, lymphosarcoma, and Hodgkin's disease. Separation methods involving prolonged treatment at relatively high temperature, such as steam distillation, have been avoided. The active substance from urine in myeloid leukemia is not volatile in steam, but although inert material can be removed by this method there is loss in activity. The number of guinea pigs used for testing each chemical fraction was always two or more. As many as thirteen guinea pigs were used for

some fractions. When a response of (+) myeloid or (+) lymphoid is mentioned in the experimental section of this paper, it indicates a positive result in all animals used for the fraction in question. The dosage was adjusted to permit a comparison of the two fractions produced by each procedure in the separation. Thus, when two fractions were obtained with 10 times as much material in the one as in the other, these proportions were maintained in the dosage for the two parts. In some cases the relative dose of the negative fraction was increased; this has been noted in the experimental section of this paper.

A method for the separation of mixtures containing both the lymphoid- and the myeloid-stimulating material has been developed. Small amounts of lymphoid-stimulating factor have been shown to occur with large amounts of the myeloid-stimulating factor in urine in myeloid leucemia. Similarly, small amounts of the myeloid-stimulating substance have been found together with large amounts of the lymphoid-stimulating material in urine in lymphoid leucemia.

The chemical separation method was applied to extracts of the urine of patients with monocytic leucemia, and Hodgkin's disease; large quantities of the two fractions producing pure myeloid and pure lymphoid biological responses were obtained from each.

The similarity in chemical properties which permits the same procedures to be used in concentrating the stimulating substances of various urines suggests strongly that the substances which produce one specific type of biological reaction are identical, regardless of their origin, and also that the myeloid- and lymphoid-stimulating factors are closely related chemically. The factor which stimulates lymphoid proliferation was found in the fraction containing hydroxy acids. This material was oxidized by two methods, one of which is specific for the conversion of carbinols to ketones. In both cases the product gave a myeloid response in animals. The myeloid-stimulating material of the non-carbinol fraction from various sources was reduced by methods adapted to the conversion of a ketone to a carbinol. The carbinols and non-carbinols were separated again; the former gave a lymphoid response, while the latter were completely negative. It seems evident from this work that the two stimulating factors for myeloid and lymphoid proliferation are acids of similar structure, differing only in the presence of a carbonyl group in the myeloid factor at the position where a hydroxyl group occurs in the lymphoid factor. Nevertheless, although it is possible to separate the lymphoid-stimulating material from impurities by virtue of its hydroxyl group, in the case of the myeloid-stimulating substance the Girard ketone separation was incomplete. This is not extraordinary (5, 6).

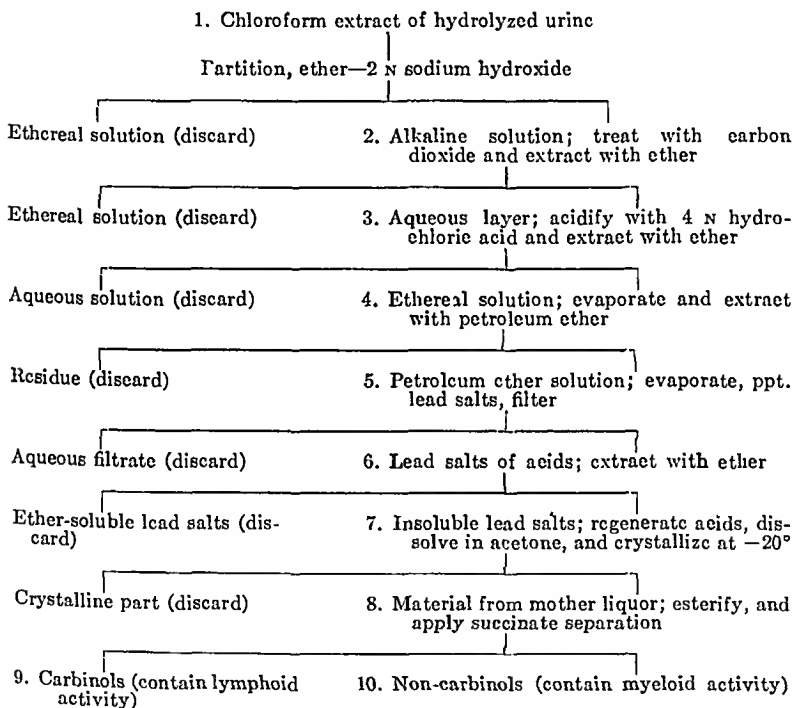
By using a method of separation similar to that employed with urine it

has also been possible to demonstrate the occurrence of myeloid-stimulating material in the feces of patients with myeloid leucemia.

### EXPERIMENTAL

*Concentration of Urine in Chronic Myeloid Leucemia*—The urine was collected from patients of both sexes with typical chronic myeloid leucemia and preserved with chloroform. It was processed in 4 liter portions. After

#### *Scheme for Concentration of Urine in Leucemia*



being adjusted to pH 1 to 2 with hydrochloric acid the acidified urine was rapidly heated to boiling and boiled for 5 to 10 minutes. It was then extracted with chloroform. The residue remaining after evaporation of the chloroform from a 360 liter portion of urine was dissolved in 1 liter of 2 N sodium hydroxide solution and extracted with ether. The neutral material remaining after evaporation of the ether gave a (—) biological response and was discarded. The alkaline solution was saturated with carbon dioxide and extracted with ether. Evaporation of the ether gave a

(-) phenolic fraction which was discarded. The aqueous layer was acidified with 4 N hydrochloric acid and extracted with ether. The ether was evaporated and the (+) myeloid residue (weight 115 gm.) was exhaustively extracted with petroleum ether (b.p. 30-60°) leaving a (-) residue. Evaporation of the petroleum ether gave nearly phenol-free acids with a (+++) myeloid response. The acid material was taken up in 2 liters of petroleum ether and extracted six times with 100 cc. portions of 90 per cent (by volume) methanol. The material soluble in methanol weighed 29 gm. It was almost biologically (-) and consisted mostly of benzoic acid identified by its melting point and melting point of mixtures with an authentic sample. The residue from the evaporation of the petroleum ether (A) weighed 21 gm. and gave a (+++) myeloid response. This was dissolved in 15 cc. of ethanol, neutralized with 40 per cent potassium hydroxide solution, and the lead salts were precipitated with a hot saturated solution of lead acetate. The mixture was cooled to 0° and filtered. The filtrate was treated with hydrochloric acid and extracted with ether. The residue, after the evaporation of the ether, gave a slight myeloid response. The lead salts were dried and extracted with ether in a Soxhlet apparatus. The regenerated acids from the ether-soluble lead salts were (-) while the regenerated acids from the ether-insoluble salts were (+++) myeloid and weighed 13 gm. This material was dissolved in 4 times its weight of acetone and crystallized at -20° (7).

The crystalline acids (weight 2 gm.) were biologically (-) and consisted of a mixture of acids, m.p. 56°, giving no depression in melting point with either palmitic or stearic acids.

*Analysis*—Found, C 75.4, H 12.6; neutralization equivalent 268

This material was evidently a mixture of palmitic and stearic acids and no attempt was made to separate it.

The mother liquor (B) from this material did not give any precipitate with digitonin. It was biologically (+++) myeloid. It was esterified with diazomethane which brought about an appreciable loss of biological activity. A 7 gm. portion of the methyl ester was dissolved in 50 cc. of pyridine and heated on the steam bath for 3 hours after 12 gm. of succinic anhydride were added. The mixture was separated into carbinols (C) and non-carbinols (D) in the usual manner (8).

The carbinol succinates (about 80 mg.) gave a (+) lymphoid reaction in animals. The non-carbinol fraction in less than a proportionate dose gave a (+++) myeloid reaction.

A 10 gm. portion of (B) was treated twice with Girard's Reagent T and once with Girard's Reagent P, the procedure being that described by Girard and Sandulesco (6). The combined ketonic fraction (about 800 mg.) gave a (++) myeloid reaction in animals. Another sample of ma-

terial corresponding to (B) was separated into ketonic and non-ketonic fractions by the method of Anchel and Schoenheimer (9), with the reagent of Borek and Clarke (10). The ketonic fraction was biologically (+) myeloid. The non-ketonic fractions from these separations were biologically (+ +) myeloid.

A sample of the free acids corresponding to (A) (6.0 gm.) was dissolved in 50 cc. of petroleum ether and adsorbed on a column 34 cm. long of 30 gm. of activated alumina (100 mesh, Aluminum Ore Company) mixed with 10 gm. of Hyflo Super-Cel. It was developed with petroleum ether until the material occupied the whole column. The column was arbitrarily divided into five fractions and these were eluted with a 50 per cent solution of acetic acid in ethanol. Material, weighing 4.7 gm., from the bottom of the column was biologically active. The residual material which accumulated in the top 10 cm. of the column was biologically inactive even with 10 times the proportionate dose.

*Concentration of Urine in Chronic Lymphoid Leucemia*—This paralleled the myeloid separation. A 170 liter portion of urine gave 55 gm. of acids soluble in petroleum ether. The activity of the fractions corresponded to that found with the myeloid extracts except that the active concentrates were specifically lymphoid in activity instead of myeloid. The petroleum ether-soluble material was subjected to the lead salt separation and here also it was possible to remove the biologically inactive ether-soluble lead salts. Decomposition of the insoluble lead salts gave 27 gm. of free acids with a (+ + +) lymphoid activity. The low temperature crystallization process removed inactive palmitic and stearic acids. The material from the mother liquor weighed 21 gm. and gave a (+ + +) lymphoid reaction in animals.

A 10 gm. portion was esterified with diazomethane. This caused some loss in biological activity. The esters were separated by the half succinate method into carbinol succinates and non-carbinols. The non-carbinols gave a (+) myeloid reaction, while the carbinol succinates (360 mg.) gave a (+ + +) lymphoid reaction even in considerably less than a proportionate dose. Hydrolysis of the half succinate esters by boiling in alcoholic potassium hydroxide gave the free acids; these were separated from succinic acid by taking advantage of the insolubility of the latter in chloroform. The product gave a (+ + +) lymphoid reaction. It was esterified with diazomethane and the esters were sublimed in a high vacuum; material subliming up to 100° (170 mg.) was only slightly active. The activity remained in the 130 mg. of residual oil.

Material corresponding to (C) from the urine of patients with acute lymphoid leucemia gave a (+ + +) lymphoid reaction, while that corresponding to (D) from this urine was inactive.

Material corresponding to (B) from the urine of five patients with lymph-

osarcoma, processed individually, gave a (++) lymphoid response in every case.

*Conversion of Myeloid Substance to Lymphoid Material*—(a) Non-carbinols (D) (4.0 gm.) from myeloid urine were hydrogenated in ether with Adams' catalyst (0.4 gm.) and hydrogen at 3 atmospheres pressure. The hydrogen absorption ceased after 1 hour. The product was separated into carbinol half succinates and non-carbinols. The former gave a (+++) lymphoid reaction, while the latter were inactive even with more than 10-fold increase of the proportionate dosage. (b) Material corresponding to (A) was reduced with sodium in ethanol. The product gave a (++) lymphoid reaction.

*Conversion of Lymphoid Material to Myeloid*—(a) Lymphoid acids (2 gm.) were neutralized with 4 N potassium hydroxide and sufficient 2 per cent potassium permanganate solution was added to the mixture at room temperature to produce a permanent pink color. The solution was decolorized by the addition of sodium bisulfite solution, acidified, and extracted with ether. Material from the ethereal solution gave a (+++) myeloid reaction in animals. (b) Lymphoid acids (7.4 gm.) were dissolved in 200 cc. of dry benzene and 60 cc. of dry acetone and refluxed for 7 hours with 12 gm. of aluminum *tert*-butylate. The product was processed in the usual manner (11). It gave a (+) myeloid reaction in animals. The ketones were separated from this material with Girard's Reagent T. They gave a (+) myeloid reaction.

*Biological Effect of Additional Reactions*—Treatment of myeloid material with phenyl isocyanate, benzoyl chloride, diethyl sulfate, or diazomethane produced only a slight loss in biological activity, if any. Oxidation with chromic acid at room temperature in acetic acid produced a partial inactivation. Treatment with bromine in acetic acid at room temperature and hydrogenation in acetic acid with Adams' catalyst effected total inactivation. Oxidation of lymphoid material with chromic acid in acetic acid at room temperature changed it to myeloid material with much loss in activity.

*Separation of Active Material from Urine in Monoeytic Leucemia and Hodgkin's Disease*—The method was the same as that employed for the myeloid urine. Material corresponding to (A) from both urines gave a peculiar reaction in animals which will be described elsewhere. This resembled the picture produced in animals by injecting both myeloid and lymphoid material together. Material corresponding to (C) from both urines gave a specific (+++) lymphoid response, while that corresponding to (D) gave a specific (+) myeloid response. The mixture corresponding to (D) from urine in Hodgkin's disease was catalytically hydrogenated as described above. The product was separated into carbinols and non-carbinols. Administration of proportionate doses of the two fractions to

guinea pigs gave a (+) lymphoid response from the carbinol fraction and a (-) response from the non-carbinols. This experiment was repeated with similar material from the urine of monocytic leucemia and the carbinol fraction from the hydrogenation gave a (+++) lymphoid response, while the non-carbinols were again (-).

*Active Material from Feces in Leucemia*—The feces of a male patient with myeloid leucemia were ground with sodium sulfate, acidified with hydrochloric acid, and extracted with methanol. The extract was hydrolyzed with alcoholic potassium hydroxide (200 gm. of potassium hydroxide in 4 liters of methanol) by boiling for 2 hours. After dilution with water, the neutral fraction was removed with ether. The alkaline layer was acidified and extracted with petroleum ether. From this point the separation followed that described for the myeloid urine. From the feces excreted in 1 week 12 gm. of crude material corresponding to (B) were obtained which gave a (++) myeloid reaction in animals.

#### SUMMARY

Concentrates of biologically active material from urine and feces of patients with leucemia are described. Urines of various diseases of the leucemia group give two active materials which produce specific myeloid or lymphoid hyperplasia in animals. Chemical methods of converting each material to the other are described.

The biological significance of this work will be discussed elsewhere. We wish to thank Dr. H. W. Jones and Dr. L. A. Erf of this laboratory for valuable assistance.

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# ISOLATION AND PARTIAL DETERMINATION OF STRUCTURE OF SOY BEAN LIPOSITOL, A NEW INOSITOL- CONTAINING PHOSPHOLIPID

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The discovery and partial purification of a new phospholipid from nerve tissue which contained inositol has been described recently by Folch and Woolley (1, 2). In order to facilitate the isolation of this new substance in pure form, a more concentrated source than nerve tissue was sought. Klenk and Sakai (3) isolated inositol from hydrolysates of crude soy bean phosphatides and hence this material was examined. Since it contained a higher percentage of inositol than crude brain phosphatides, it was used as starting product for the isolation of the new lipid in the hope that the phospholipid from this plant source would be the same as that in nerve tissue.

Folch's (2) method of obtaining an inositol-containing fraction from brain cephalin by precipitation from chloroform with alcohol was applied to soy bean phosphatides. Purification was achieved by several fractional precipitations with methanol and ethanol, followed by extraction with ether. This method yielded a product containing 16 per cent inositol. The inositol content could not be increased by additional fractionation. Furthermore, the molecular ratio of inositol, carbohydrate, phosphorus, and oleic acid was approximately unity. For these reasons it was believed to be essentially pure.<sup>1</sup>

It is proposed to call the new substance soy bean lipositol, since it is a lipid which contains inositol. For reasons given below, it is concluded that it is very similar to the phospholipid of brain and spinal cord.

The products of complete acid hydrolysis of soy bean lipositol were identified as inositol, phosphoric acid, oleic acid, saturated fatty acids, a black, humin-like material, traces of ethanolamine and tartaric acid, and a substance which yielded ethanolamine on hydrolysis with alkali. The saturated fatty acids were composed of 5 per cent cerebronic acid and 95 per cent of a mixture of approximately 70 per cent palmitic acid and 30 per cent stearic acid.

\* With the technical assistance of A. G. C. White.

<sup>1</sup> Since the saturated fatty acid component was a mixture, the lipositol must have been a mixture of three homologues. Therefore, "pure" lipositol denotes a product free of other phosphatides, and not one single compound.

Less complete acid hydrolysis resulted in the formation of inositol monophosphate. This confirmed the observation of Klenk and Sakai (3), and led to the assignment of the position of the phosphorus atom in the molecule. During acid hydrolysis, practically all of the inositol was rendered water-soluble within the 1st hour, but all of it was not free inositol until after further hydrolysis. The fact that yeast responded only to free inositol and not to inositol esters (4) facilitated the elucidation of these facts. The water-soluble, bound inositol was presumably inositol monophosphate. This behavior during acid hydrolysis was found for the lipositol of brain as well as for pure soy bean lipositol.

The products of hydrolysis with alkali were fatty acids (presumably the same as those liberated by acid), ethanolamine, *d*-tartaric acid, a non-reducing carbohydrate, and phosphoric acid. On mild acid hydrolysis, the carbohydrate liberated inositol, tartaric acid, and galactose in the molecular ratio of 1:1:1.

TABLE I  
*Constituents of Soy Bean Lipositol*

Constituent	Found		Constituent	Found	
	By iso-	By		By iso-	By
	lation	ana-		lation	analysis
	per cent	per cent		per cent	per cent
Inositol.....	15	16	Oleic acid .. . . . . .	23.6	
Galactose.....		15.5	Phosphoric acid.....		9.8
Tartaric acid.....	8.3		Saturated acids .. . . .	21.2	
Ethanolamine.....	0.44		Potassium.....		3.4

The products of hydrolysis which have been isolated or determined quantitatively account for 102 per cent of the lipositol (see Table I). The hydrolysis products should account for 109 per cent of the substance, since water is added during hydrolysis. It is felt that all constituents of the molecule have been accounted for.

Lipositol as isolated from soy beans was the potassium salt of a strong acid. It was not possible to prepare the free acid, for the ash content was not altered by dialysis from suspension in HCl, or by extraction of lipositol with ether from suspension in HCl.

The position of some of the radicals in the molecule was deduced from a study of the products of various hydrolyses. The allocation of the phosphate to inositol followed from the isolation of inositol monophosphate. Since hydrolysis with alkali yielded a non-reducing carbohydrate which liberated reducing sugar on acid hydrolysis, the galactose must be present as a galactoside. Either a hydroxyl of inositol or tartaric acid could

be involved in this galactoside linkage. But since hydrolysis with aqueous alkali yielded as much tartaric acid as could be obtained by a combination of alkali and acid hydrolysis, and since reducing sugar was only set free by acid, it was concluded that an inositol galactoside was present in the molecule. Ethanolamine was only set free by hydrolysis with alkali. Hydrolysis with acid produced a water-soluble nitrogenous substance which gave rise to ethanolamine when heated with alkali. Finally, tartaric acid was found in appreciable quantities only after hydrolysis with alkali. Therefore, it was concluded that ethanolamine tartrate was present in the molecule, and that this ester was not readily hydrolyzed under the acid conditions used. These observations may offer an explanation for the non-ethanolamine amino nitrogen in crude phosphatides described by Chargaff *et al.* (5), since their hydrolyses were performed with acid. In lipositol, the other carboxyl of tartaric acid was probably esterified with a hydroxyl of inositol or galactose. At least one more hydroxyl of inositol (in addition to the ones carrying phosphoric acid and galactose) must be esterified with fatty acid or tartaric acid and any unsubstituted hydroxyls must not be adjacent. This followed from the fact that lipositol was not split rapidly by lead tetraacetate or by periodic acid. These reagents react immediately with hydroxyls situated on adjacent carbon atoms and cause rupture of the carbon chain. The reaction with these reagents was measured by determining the inositol remaining after the reaction. A slow but complete destruction did occur in 24 hours in the presence of a large excess of the reagents, but since 50 per cent of the inositol contained in inositol hexaacetate was destroyed under similar conditions, it was felt that the above conclusions were valid.

From the present data, lipositol may be considered to consist of inositol with which phosphoric acid, oleic acid, a saturated fatty acid (chiefly palmitic acid), and ethanolamine tartrate have been esterified and galactose has been combined in glycosidic linkage. One or two, but not more, of the carboxylic acids may be esterified with galactose rather than with inositol. The percentages of the elements and of most of the products of hydrolysis agree reasonably well with such formulation. However, because of lack of data concerning relative positions of all constituents, the complete structure of lipositol is not known.

Pure lipositol from brain has not yet been isolated, but the following points of similarity to soy bean lipositol were noted. First, the rate of liberation by acid of inositol phosphate and of inositol in a preparation of cephalin from brain was the same as from soy bean lipositol. Secondly, the ratio of inositol to carbohydrate in this same preparation from brain was 1:1, the same as in lipositol. Finally, tartaric acid was isolated from hydrolysates obtained by the use of alkali.

## EXPERIMENTAL

*Isolation of Lipositol*—The following isolation has been performed fourteen times with uniform success. The attention to conditions of drying was very important, since perfectly dry lipositol was insoluble in all fat solvents tested (ether, chloroform, benzene, petroleum ether), although it was readily soluble in the moist solvents. Inositol was determined according to Woolley (6) after hydrolysis for 24 hours, and the degree of purification was deduced from the inositol content of the various fractions.

200 gm. of commercial soy bean phosphatides<sup>2</sup> (3 per cent inositol) were dissolved in 200 cc. of chloroform and poured into 1 liter of methanol. The precipitate which formed was separated and dissolved in 200 cc. of chloroform and poured into 1200 cc. of acetone. The precipitate was filtered off and washed with acetone, and then dissolved in 200 cc. of chloroform. This solution was poured into 800 cc. of methanol, and the precipitate was reprecipitated in the same fashion. The precipitate was next taken up in 150 cc. of chloroform and poured into 300 cc. of absolute ethanol. The precipitate was separated in a centrifuge and redissolved in 150 cc. of chloroform. The solution was poured into 200 cc. of absolute ethanol, and the precipitate again collected in a centrifuge, and redissolved in 200 cc. of chloroform. This solution was added to 800 cc. of methanol, and the precipitate was filtered, washed with methanol, and freed of solvent in a moist atmosphere.<sup>3</sup> It was then suspended in 200 cc. of chloroform, and, when all lumps had disintegrated, 100 cc. of absolute ethanol were added. The precipitate was removed in a centrifuge, and the supernatant was treated with 150 cc. of absolute ethanol. The precipitate which formed was collected in a centrifuge, dissolved in 200 cc. of chloroform, and the solution was poured into 600 cc. of methanol. The precipitate was filtered off, washed with methanol, and freed of solvent in a moist atmosphere. It was then suspended in 400 cc. of ether and the suspension was centrifuged until perfectly clear. The supernatant fluid was finally poured into 4 volumes of methanol, the suspension was filtered, and the precipitate was washed with methanol and dried in a vacuum. Yield, 20 to 30 gm.

*Properties of Lipositol*—The phospholipid was obtained as a fine, creamy, white powder which, when dry, was quite friable. It darkened only very

<sup>2</sup> We wish to thank Dr. A. Scharf, of the American Lecithin Company, for a supply of commercial soy bean phosphatides.

<sup>3</sup> This was accomplished in one of two ways. The filter cake was placed in a desiccator which contained a dish of water and the desiccator was frequently evacuated. Usually the filter cake was allowed to dry in air of relative humidity approximately 80 and at 25°. This second procedure did not result in appreciable darkening of the product, since the easily oxidizable compounds were removed by the previous precipitations with ethanol.

slowly when exposed to air. However, if no precautions were taken to prevent oxidation<sup>4</sup> during preparation, the final product was light brown. It was insoluble in dry petroleum ether, dry acetic acid, dry benzene, dry ether, or dry chloroform, but soluble in any of these solvents except acetic acid when moist. It was insoluble in ethanol, methanol, acetone, and dioxane, but formed an emulsion easily with water. Found, C 54.3, H 8.4, P 3.1, N 0.98, ash 12.1, K 3.4, inositol 16, carbohydrate 15.5, amino N 0.80.

Carbohydrate was determined according to Sørensen and Haugaard (7). The amino N was determined on hydrogenated material in order to avoid interference and high values due to unsaturated fatty acids. The total N was not changed significantly in the hydrogenated product.  $\alpha$ -Amino acid N (8) was absent.<sup>5</sup>

*Rate of Liberation of Inositol by Acid*—20 mg. samples were emulsified in 20 cc. of 20 per cent HCl and the emulsions were refluxed for stated times. The insoluble matter was filtered off and inositol was determined without

TABLE II

*Rate of Liberation of Free and Total Inositol during Hydrolysis of Lipositol with 20 Per Cent HCl*

Time of hydrolysis	Water-soluble inositol	
	Free	Total
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>
0.5	5.0	9.8
1.0	8.2	13.5
6	16	16
24	16	16

further hydrolysis in an aliquot of the filtrate (free inositol). A second aliquot was refluxed for 24 hours and inositol determined in it. This value gave the total water-soluble inositol. Results are given in Table II. It can be seen that practically all the inositol was made water-soluble in the 1st hour, but that it was not all free (and available to yeast) until after about 6 hours.

<sup>4</sup>Precautions to avoid oxidation were the use of closed vessels filled almost completely by the solutions in order to minimize exposure to air, the use of low temperatures (4°) in the first precipitation, and rapid manipulation. The easily oxidizable substances were removed in the early steps and precautions against oxidation were relaxed after the precipitations with ethanol. A product without color was obtained readily by application of the procedure to a cold ether extract of fresh soy beans, but the yield was not great enough to justify use of fresh soy beans as the starting material.

<sup>5</sup>We wish to thank Dr. J. Folch, of the Hospital of The Rockefeller Institute for Medical Research for this analysis.

*Products of Acid Hydrolysis*—20 gm. of pure lipositol were suspended in 400 cc. of 20 per cent HCl and the emulsion was refluxed for 20 hours. The mixture was cooled and filtered.

*Fatty Acids*—The water-insoluble portion was extracted with acetone and filtered. The acetone was removed from the solution under reduced pressure, the residue was extracted with petroleum ether, and the extract filtered and dried under reduced pressure. 9 gm. were obtained. 3.4 gm. of the product were separated by the lead soap method (9) into 1.6 gm. of saturated acids, and 1.8 gm. of unsaturated acid.

*Oleic Acid*—The unsaturated acid was pure oleic acid, for the following reasons. The iodine number (Hanus) was 90.2; that for oleic acid is 90.0. When the acid was hydrogenated in acetic acid with Pt catalyst, it yielded stearic acid, m.p.<sup>6</sup> 68°, mixed m.p. with stearic acid 68°.

*Cerebronic Acid*—The saturated acids were converted to Mg salts and separated according to the method of Klenk (10). The hot alcohol-insoluble salt was converted to the free acid which melted at 98–100°.

$C_{21}H_{40}O_2$ . Calculated, C 75.0, H 12.5; found, C 74.6, H 12.4

The neutral equivalent was 378. The acetate prepared with acetyl chloride melted at 56–57°. 500 mg. of one preparation of saturated acids yielded 25 mg., and 1.6 gm. of another preparation from a new sample of lipositol yielded 80 mg. of cerebronic acid. The saturated acids were therefore 5 per cent cerebronic acid.

*Palmitic and Stearic Acids*—The acids regenerated from the soluble Mg salts melted at 53° and three recrystallizations from acetone did not alter this value. While a pure acid was not isolated from this mixture, the following facts indicated that it was a mixture of approximately 70 per cent palmitic acid and 30 per cent stearic acid. Found, C 75.4, H 12.5. The ratio of C to O was therefore 16.6:2. A mixture of 7 parts of palmitic to 3 of stearic acid melted at 53°, and admixture of this mixture with the acids from lipositol caused no depression of the melting point.

It was clear that saturated and unsaturated acids were in equimolecular ratio in lipositol, and that there was approximately 1 molecule of oleic acid per molecule of inositol per molecule of saturated acid.

*Inositol*—The water-soluble portion of the hydrolysate was concentrated under reduced pressure to a sirup, and this residue was extracted with 100 cc. of absolute ethanol. The crystals which formed were filtered off and recrystallized from dilute alcohol. Yield, 3.0 gm.; m.p. 218°. The crystals gave Seherer's test for inositol and were fully active in the highly specific test for mesoinositol (6).

$C_6H_{12}O_6$ . Calculated, C 40.0, H 6.7; found, C 39.9, H 6.7

<sup>6</sup> All melting points recorded in this paper are uncorrected.

*Inositol Monophosphate*—10 gm. of lipositol were suspended in 200 cc. of 20 per cent HCl and the emulsion was refluxed for 1 hour. The fatty acids were filtered off and washed, and the aqueous hydrolysate was concentrated under reduced pressure to dryness. The residue was dissolved in 100 cc. of water and treated with excess lead acetate solution. The precipitate was filtered off, washed, suspended in water, and decomposed with  $H_2S$ . The filtrate from the lead sulfide was concentrated under reduced pressure to dryness, and this residue was triturated with 100 cc. of absolute ethanol. The amorphous precipitate which formed was filtered off and purified by precipitation from concentrated aqueous solution with absolute ethanol. It was obtained as 1 gm. of fine, white, slightly hygroscopic powder.

$C_6H_{11}O_5P$ . Calculated, inositol 69; found, inositol 69

The brucine salt was prepared and recrystallized from dilute acetone as fine needles; m.p.  $238^\circ$ , after softening at  $218-220^\circ$ . Inositol monophosphate brucine salt is said to melt at  $236^\circ$  (3).

The barium salt was precipitated by alcohol from aqueous solution as a white amorphous powder which contained 35.4 per cent Ba; theory for  $C_6H_{11}O_5PBa$ , 34.7.

An attempt was made to isolate barium glycerophosphate from the alcohol filtrate of inositol monophosphate. 300 mg. of an alcohol-insoluble barium salt were obtained whose barium content was that of barium inositol monophosphate.

*Attempted Isolation of Ethanolamine*—The filtrate from the lead precipitate obtained in the previous operation was freed of lead with  $H_2S$ . Analyses showed that 80 per cent of the nitrogen originally present in the lipositol was contained in this solution, and that all of it was primary amino nitrogen. The solution was concentrated, dried on  $CaO$ , and extracted in a Soxhlet apparatus with an ether solution of 3,5-diiodosalicylic acid (11). Only 7 mg. of ethanolamine 3,5-diiodosalicylate, m.p.  $198^\circ$ , were obtained. In a second experiment, the hydrolysate was prepared from 5 gm. of lipositol, according to the directions of Chargaff (11), but only 2 mg. of ethanolamine 3,5-diiodosalicylate were obtained. In order to establish that there was no material present which interfered with the isolation of the ethanolamine salt, 100 mg. of ethanolamine were added to 5 gm. of lipositol, and the procedure described above was repeated. Ethanolamine 3,5-diiodosalicylate, equivalent to 22 mg. of ethanolamine, was isolated. It was therefore concluded that only traces of ethanolamine were present in acid hydrolysates of lipositol.

After hydrolysis with alkali of an acid hydrolysate of lipositol, ethanolamine was isolated. 5 gm. of lipositol were hydrolyzed with HCl as described above, and the aqueous hydrolysate was concentrated under reduced pressure to a sirup. This was neutralized with NaOH and mixed with 20



cc. of  $N$  NaOH. The solution was refluxed for 4 hours, acidified with HCl, and concentrated under reduced pressure to a sirup, which was extracted with alcohol. The alcohol extract was concentrated, made alkaline, dried on plaster of Paris, and extracted in a Soxhlet apparatus with an ether solution of 3,5-diiodosalicylic acid for 48 hours. 100 mg. of ethanolamine 3,5-diiodosalicylate, m.p.  $198^{\circ}$ , were obtained.

*Attempted Isolation of Tartaric Acid from Acid Hydrolysates*—2 gm. of lipositol were hydrolyzed with 20 cc. of boiling  $2 N$  HCl for 4 hours. The aqueous portion of the hydrolysate was concentrated under reduced pressure, dissolved in 20 cc. of water, and extracted with ether for 90 hours. Only 28 mg. of solid matter, neutral equivalent 75, were contained in the extract. Since large amounts of tartaric acid were present in ether extracts of hydrolysates prepared with alkali (see below), it was concluded that the acid hydrolysis had not liberated much tartaric acid.

*Hydrolysis with Alkali*—10 gm. of lipositol were dissolved in 50 cc. of moist ether and poured into 150 cc. of methanol which contained 6 gm. of NaOH. The suspension was refluxed for 5 hours, cooled, filtered, and the precipitate was washed with methanol.

*Ethanolamine*—The methanol-soluble hydrolysate was acidified with concentrated HCl, and the NaCl which formed was filtered off and washed. The filtrate was concentrated under reduced pressure to an oil which was heated with 100 cc. of water. The fatty acids were filtered off and washed, and the aqueous phase was extracted twice with ether, and then concentrated under reduced pressure to 2 cc. This solution was dried on CaO and extracted in a Soxhlet apparatus with an ether solution of 1 gm. of 3,5-diiodosalicylic acid for 48 hours (11). 326 mg. of ethanolamine 3,5-diiodosalicylate, m.p.  $198^{\circ}$ , were obtained.

$C_{21}H_{31}O_4I_2N$ . Calculated, N 3.1; found, N 3.1

The salt was decomposed by suspending it in  $N$  HCl and extracting the suspension with ether, and the free base was recovered by drying the aqueous phase on CaO and extracting with ether for 48 hours in a Soxhlet apparatus. When picric acid was added to the free base, a picrate was formed which melted at  $154^{\circ}$ . In the same bath, ethanolamine picrate melted at  $154$ – $155^{\circ}$ . The mixed melting point was  $154^{\circ}$ . The benzoyl derivative was formed with benzoyl chloride and NaOH, and was found to melt at  $76^{\circ}$ . Benzoylated ethanolamine in the same bath melted at  $76^{\circ}$ . The flavianate was prepared and found to melt at  $203$ – $204^{\circ}$ , while ethanolamine flavianate in the same bath melted at  $204^{\circ}$ .

The ethanolamine isolated did not account for all the nitrogen of lipositol. However, since it was only possible to recover 20 to 25 per cent of ethanolamine added to lipositol, it was concluded that more was present than was isolated.

*Tartaric Acid*—The methanol-insoluble portion of the hydrolysate was suspended in 100 cc. of water, acidified with acetic acid, and centrifuged. The clear solution was neutralized (pH 6.5) with NaOH and treated with excess lead acetate solution. The precipitate which formed was centrifuged out, washed, and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the lead sulfide was concentrated under reduced pressure to 20 cc. and extracted continuously with ether for 90 hours. The extract was concentrated to dryness to leave a white crystalline mass which weighed 300 mg. Neutral equivalent, 74.

The silver salt was prepared and found to contain 59.5 per cent Ag; theory for silver tartrate is 59.3 per cent Ag. The calcium salt was prepared and found to contain 21.6 per cent Ca; theory for calcium tartrate is 21.3 per cent Ca. In addition, the acid gave those color tests which have been described for tartaric acid (12); that is, a blue color with  $\text{FeSO}_4$ ,  $\text{H}_2\text{O}_2$ , and NaOH, and a green color with  $\beta$ -naphthol. Finally, the brucine salt was prepared and recrystallized to constant melting point from dilute acetone. It darkened at about  $220^\circ$ , and melted and decomposed at  $240^\circ$ . Brucine *d*-tartrate in the same bath behaved in similar fashion. The tartaric acid was not optically pure, for  $[\alpha]_D$  was found to be  $+9.2^\circ$  (tartaric acid showed  $+12^\circ$  under similar conditions), but it is probable that some racemization had occurred during hydrolysis with alkali.

Combined tartaric acid was present in the lead filtrate obtained above. It could be freed by heating with either aqueous acid or alkali, and could then be precipitated with lead or barium and extracted with ether. In this way, a further yield of 330 mg. was isolated. The total yield of tartaric acid was therefore 630 mg. (6.3 per cent of lipositol). A slightly higher yield (8.3 per cent) was obtained by acid hydrolysis of a hydrolysate obtained with alkali, followed by direct extraction with ether.

*Inositol Galactoside Tartrate* (?)—A lead filtrate obtained (as described above) during the isolation of tartaric acid was treated with 2 gm. of lead acetate and excess ammonia, and the precipitate which formed was collected and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the lead sulfide was concentrated under reduced pressure to an oil which was triturated with methanol. The white powder which formed was purified by reprecipitation from water with methanol. Yield, 1.2 gm. It contained 33 per cent inositol (by analysis), 33 per cent carbohydrate (by analysis (7)), 26 per cent tartaric acid (by isolation). It was acidic (neutral equivalent approximately 500) and non-reducing. As much tartaric acid was liberated by aqueous alkali as by acid. It was believed to be inositol galactoside tartrate. Since hydrolysis with normal aqueous NaOH yielded tartaric acid and a substance which liberated inositol and galactose on acid hydrolysis, it was believed that this was a partial hydrolysis product which had escaped the action of the alcoholic alkali owing to its low solubility in this solvent.

*Galactose*—The methanol-insoluble hydrolysate of 10 gm. of lipositol, obtained after hydrolysis with alkali as described above, was taken up in 40 cc. of  $N H_2SO_4$  and refluxed for 4 hours. The solution was chilled, adjusted to pH 8 to 9 with cold barium hydroxide, and the precipitate was removed. The solution was acidified with  $H_2SO_4$ , treated with an excess of  $BaCO_3$ , filtered, and concentrated under reduced pressure to a sirup. This was extracted twice with methanol. Inositol was identified in the alcohol-insoluble material by its melting point ( $218^\circ$ ) and the melting point of its acetate ( $212^\circ$ ). The methanol-soluble material was freed of solvent, taken up in water, and used for sugar tests.

Yeast did not ferment the sugar (1 per cent solution based on total carbohydrate content), and this failure was not due to inhibition of the yeast, for, when glucose was added to the solution, it was fermented as rapidly as was

TABLE III

*Destruction of Inositol in 20 Mg. of Lipositol by Lead Tetraacetate and Periodic Acid*

Lead tetraacetate	Periodic acid	Time of reaction	Inositol destroyed
mg.	mg.	hrs.	per cent
40		1	44
100		20	81
1000		0.25	84
1000		24	100
	40	1	69
	50	0.25	89

pure glucose. Mannose was not present, for no phenylhydrazone could be obtained and, when mannose was added to the solution, as much phenylhydrazone was isolated as from a solution of the same quantity of mannose.

When heated with phenylhydrazine, an osazone was formed which melted at  $200-202^\circ$ . Galactose phenylosazone melted at  $198-200^\circ$ . The solubility behavior and crystal habit of the osazone closely resembled those of galactose phenylosazone.

A portion of the solution which contained 300 mg. of reducing sugar (calculated as glucose (13)) was oxidized and separated according to Moore and Link (14). The aldonic acid was all found in the insoluble K salt fraction. This K salt was converted to the benzimidazole according to the Moore and Link procedure (14). 356 mg. of product were obtained which, after recrystallization, melted at  $244^\circ$ . The picrate of this base melted at  $215^\circ$ , and at  $215-216^\circ$  when mixed with galactose benzimidazole picrate. The benzimidazole of galactose and its picrate are said to melt at  $245^\circ$  and  $217^\circ$  respectively (14).

*Oxidation with Lead Tetraacetate and Periodic Acid*—20 mg. samples of lipositol were dissolved in 10 cc. of chloroform and treated with lead tetraacetate. At the end of the reaction time, the solvent was removed under reduced pressure, water was added, and  $H_2S$  was immediately passed into the solution. Hydrolysis and determination of inositol were carried out as usual.

For the experiments with periodic acid, the 20 mg. samples of lipositol were suspended in water and treated with  $HIO_4$ . To stop the reaction, excess glycerol was added. As can be seen from the data in Table III, the inositol was destroyed completely only after a considerable reaction time.

#### SUMMARY

A method has been described for the isolation of lipositol, an inositol-containing phospholipid, from soy bean phosphatides. The compound contained 16 per cent inositol, 15.5 per cent carbohydrate identified as galactose, 8.3 per cent *d*-tartaric acid, 23.6 per cent oleic acid, a mixture of cerebronic, palmitic, and stearic acids equivalent in amount to the oleic acid, phosphoric acid, and ethanolamine. Isolation of some products of partial hydrolysis and a consideration of some of their properties have led to conclusions regarding the structure of the molecule. Some properties of lipositol have been described.

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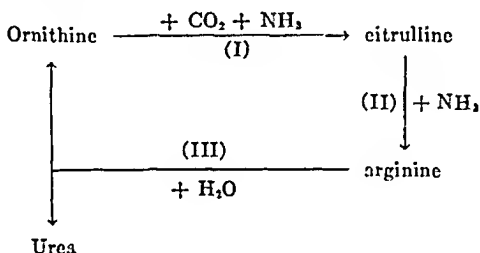
# THE SYNTHESIS OF UREA IN THE LIVER, WITH SPECIAL REFERENCE TO CITRULLINE AS AN INTERMEDIARY IN THE ORNITHINE CYCLE\*

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In the theory of Krebs and Henseleit (1-3) the production of urea in the liver is the outcome of the recurrent cycle of reactions represented by the accompanying scheme.



Of the three consecutive reactions here postulated, each hereinafter designated by its appended numeral, two (I and II) depend on the presence of actively respiring liver cells, while (III) is effected by the soluble enzyme arginase. The probability that reaction (I) takes place in two stages (3) may, for the purposes of this paper, be neglected. If the theory be correct, then so long as the cells have an adequate supply of  $\text{NH}_3$  and  $\text{CO}_2$  (together with oxygen and a suitable respiratory substrate) a small quantity of ornithine will "catalyze" the formation of an indefinite quantity of urea. The discoveries (a) that ornithine does act in this way and (b) that among other amino acids citrulline alone has a comparable effect upon urea synthesis constituted the basis on which the theory was formed, and remain the principal items of evidence in its support.

As additional evidence for the rôle which the theory assigns to citrulline Krebs and Henseleit claimed that, whereas with ornithine as catalyst the ratio of ammonia nitrogen used up to urea nitrogen produced was approximately 1 (0.91), with citrulline it was more nearly 0.5 (0.69). Bach (4) failed to confirm this observation, finding with citrulline a ratio of 1, and

\* The experimental data of this paper are taken from a thesis presented by Allan G. Gornall in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Toronto.

he used this failure as evidence that the path from citrulline to urea does not necessarily pass through arginine. Whatever may be the bearing of the ratio upon the mechanism of urea formation—a point we shall leave for later discussion—it is evident that the question of its actual magnitude merits further study.

Although both ornithine and citrulline have been recognized as promoters of urea synthesis, little attention has hitherto been paid to their relative efficiencies in this regard. In so far as the point has been touched upon, citrulline has seemed to be the more effective catalyst (1, 4, 5). In view of the paucity of experimental data, it seems desirable that this aspect of the matter, theoretically of no little interest, should be more thoroughly investigated.

The validity of the hypothesis of Krebs and Henseleit would be greatly enhanced if citrulline could be isolated from liver tissue or if either reaction (I) or (II) could be shown actually to occur as an accompaniment of urea synthesis. It is quite certain that animals can in some manner convert ornithine into arginine (6), and also that the amidine group of the latter (the part liberated by arginase as urea) can be built up out of  $\text{NH}_3$  (7) and  $\text{HCO}_3^-$  (8, 9). Kitsugawa (10), perfusing the wall of the stomach, obtained some evidence for the conversion of citrulline into arginine or (presumably through the successive operation of reactions (II) and (III)) into ornithine. Ikeda (11) has reported the production of ornithine from citrulline in the perfused liver. Borsook and Dubnoff (12) have demonstrated that surviving kidney slices can transform citrulline into arginine. On the other hand for the occurrence of reaction (I), or indeed for the production under any circumstances of citrulline in the animal body, there has hitherto been no direct evidence.

With the foregoing considerations in mind we have carried out, and now report, the following three groups of experiments: (1) an extended series in which ornithine and citrulline, in a wide range of concentrations, were compared with respect simply to their effect upon the speed of urea synthesis. This series included also a number of experiments with arginine; (2) a group demonstrating the actual production of citrulline from ornithine during the synthesis of urea; (3) a group devoted to a comparison of the ammonia nitrogen to urea nitrogen ratios exhibited under the catalytic influence of widely varied concentrations both of ornithine and of citrulline.

In actual performance these three groups of experiments partly overlapped one another. The general outcome of all was to confirm, and in certain respects to amplify, the theory of Krebs and Henseleit.

### *Methods*

*General Technique*—In all of our experiments we employed the tissue slice technique, adopting, with only minor modifications, the procedure

described by Krebs and Henseleit (1). The animals used were Wistar rats, of either sex, killed by stunning after a fasting period of, usually, 48 hours. The livers were cut free-hand with a thin razor blade, and slices, trimmed about 30 sq. mm. and of 0.3 mm. thickness, were washed thrice, for 2 minutes at a time, in 20 ml. of a 0.9 per cent NaCl solution, to each liter of which there had been added 20 ml. of 1.15 per cent KCl and 20 ml. of 0.11 M  $\text{CaCl}_2$ . The washed slices, in a number (five to seven) estimated to have a dry weight of about 10 to 12 mg., were distributed in 25 ml. Erlenmeyer flasks already charged with 3.5 to 4.5 ml. of the required reaction mixture. The flasks, each of which was provided with an inlet and a capillary outlet, were immersed, on a multiple holder, in a water thermostat at 37.5°. The inlets were connected through a manifold and a wash bottle to a reservoir of oxygen containing 5 per cent of  $\text{CO}_2$ ; the outlets dipped just below the surface of the bath. The flasks were shaken at a rate of 70 cycles per minute with an amplitude of 6 cm. For the first 12 minutes or so they were flushed with the gas mixture at a rate to cause vigorous bubbling from the outlets. After this period of equilibration the flow of gas, though not completely interrupted, was cut down to about twenty bubbles per minute. The experiment was timed from this point, which was reached in less than 1 hour from the killing of the animal. The duration of the experiment was usually 2 hours. At its completion the liver slices of each vessel were removed, rinsed, dried, and weighed. Convenient fractions of the residual medium were taken for the determination of urea, citrulline, and ammonia.

*Reaction Mixture*—This consisted of the "new" physiological saline of Krebs and Henseleit (1), with admixtures of  $\text{NH}_3$ , lactate, and, as the case might require, either ornithine or citrulline. Its volume varied in different experiments between 3.5 and 4.5 ml. The  $\text{NH}_3$  was supplied in the form of a 0.827 per cent  $\text{NH}_4\text{Cl}$  solution, and in such amount as to give in the completed mixture an  $\text{NH}_3$  concentration of 0.015 per cent. For the lactate, we have used both commercial and crystallized lactic acid, neutralized with NaOH to pH 7.4, and diluted to give a 3 per cent solution of sodium lactate. The volume taken was that required to give a final concentration of 0.2 per cent. *l*(+)-Ornithine hydrochloride and *l*(+)-citrulline, prepared according to Hunter (13) and Gornall and Hunter (14) respectively, were used in concentrations so adjusted (from 0.2 up to 5.4 per cent) that the volume of amino acid solution required was usually about one-twentieth and never more than one-fifth of the whole reaction mixture. The saline, in quantity sufficient to make up the total volume desired, was added last. Reference to the sample protocol of Table I will clarify the procedure. The pH of the final mixture was always 7.4. The dilution effected by the addition of the moist liver slices was estimated at not more than 1 or 2 per cent.



*Determination of Ammonia*—In our first experiments, taking for each determination 0.5 ml. of the reaction mixture, we employed for ammonia the photocolorimetric micromethod of Mason and Rozzell (15), which depends on nesslerization. Later we discovered that the development of the Nessler reaction is hindered by the presence of ornithine. This phenomenon, which we do not remember to have seen mentioned before, can lead to serious error. In mixtures containing 0.5 per cent of ornithine, a concentration exceeded in some of our experiments, the color produced from ammonia is about half as intense as would normally be expected. Only with ornithine concentrations less than about 0.025 per cent can this effect be safely neglected. Citrulline shows an interference similar in kind, but much less marked, becoming perceptible only at concentrations above 0.1 per cent and involving no serious error even at 0.25 per cent.

The disturbing effect of ornithine was avoided in later experiments by adopting for ammonia the diffusion-titration method of Conway and Byrne (16). The volume of reaction mixture taken for the estimation was usually 0.7 ml., containing 40 to 80  $\gamma$  of  $\text{NH}_3$ . Over this range the method gave in control experiments results within 2 per cent of the theoretical.

*Determination of Urea*—This was carried out on 3 ml. of the mixture by the manometric urease method of Krebs and Henseleit (1). The urease, in order that it should interfere as little as possible with the subsequent determination of citrulline, was prepared (from an extract of jack bean meal) by a double precipitation with acetone (17). In use, 1 gm. of the dry enzyme powder was digested for an hour with 10 ml. of water. The resultant suspension was treated with 0.5 ml. of acetate buffer (1), allowed to stand for a further half hour, and then lightly centrifuged. The supernatant liquid, if kept in the ice box, retained its activity for at least 2 weeks.

*Determination of Citrulline*—After the determination and destruction of urea the contents of the manometer vessel were transferred quantitatively to a 15 ml. volumetric flask, cleared of protein, and analyzed for citrulline. We have described elsewhere (17) the photocolorimetric method employed and the details of its application to tissue slice experiments.

#### *Comparison of Ornithine and Citrulline in Their Effects upon Urea Synthesis*

In experiments upon over 80 rats, involving more than 300 individual determinations of urea, we studied the production of that substance by liver slices from fasted animals in a saline medium containing, per 100 ml., 15 mg. of  $\text{NH}_3$ , 200 mg. of sodium lactate, and varying quantities (from 0.5 to 1000 mg.) of either ornithine or citrulline or (in a few cases) arginine. About one-third of the measurements was made under the general conditions represented in Table I, the effects of ornithine and citrulline being

compared in parallel experiments upon slices from a single liver. In the remainder one catalyst only was studied at a time. For each liver made use of we determined the basal level of urea synthesis by a control experiment, in which the saline bathing the tissue slices had no other additions than ammonia and lactate. The duration of the experiment was in every case 2 hours.

The results of the urea determinations are expressed in terms of the "urea quotient" of Krebs and Henseleit (1); *i.e.*,  $Q_{\text{urea}} = (\text{c.mm. of CO}_2 \text{ from urea})/(\text{mg. of dry tissue} \times \text{hours})$ . The effect of an added "catalyst" upon urea synthesis is measured by the increase of the quotient above the

TABLE I  
Sample Protocol. Rat 101

Volume of reaction mixture, 4.5 ml.;  $\text{NH}_3$  0.015 per cent, Na lactate 0.2 per cent; time, 2 hours.

Vessel No.....	1	2	3	4	5	6	7
$\text{NH}_4\text{Cl}$ 0.827%, ml.....	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Na lactate 3%, ".....	0.30	0.30	0.30	0.30	0.30	0.30	0.30
l(+)-Ornithine HCl, 2.6%, ml. ....		0.03	0.13				
"    "    5.2%, "    "    .....				0.33			
l(+)-Citrulline, 2.7%, ml.....					0.03	0.13	
"    "    5.4%, "    "    .....							0.33
Saline, ml.....	3.94	3.91	3.81	3.61	3.91	3.81	3.61
Ornithine, mg. per 100 ml.....	0	17.5	75	380			
Citrulline, "    "    100 ".....	0				18	78	396
Dry weight of slices,* mg.....	10.71	10.47	10.96	11.33†	10.72	11.03	11.13
Urea in 2 hrs., c.mm. $\text{CO}_2$ .....	112.8	222	227.3	165.0	218	237	200
"    quotient.....	5.27	10.60	10.37	7.28†	10.17	10.74	8.99
"    "    increase.....		5.33	5.10	2.01†	4.90	5.47	3.72

\* Seven slices in each.

† Mean values from two parallel experiments.

basal value indicated by the control. Our basal quotients, ranging mostly between 4.0 and 6.0 and averaging 5.2, have, generally speaking, been larger than those reported by Krebs and Henseleit, our gross quotients of similar magnitude to theirs, and our observed increases therefore on the whole somewhat smaller.

To display the numerical data in their entirety would take an undue amount of space. We content ourselves therefore with reporting (a) in Table I the complete protocol of a single representative experiment, (b) in Table II a list of the individual values for increase of urea quotient found, anywhere in the series, at one particular concentration (10 mg. per cent) of ornithine or citrulline, and (c) in Table III a summary of the col-

lected results at all concentrations of ornithine, citrulline, and arginine tested. In the construction of the summary a few experiments, which (like those of Table I) were made at concentrations other than those specified, have been grouped for convenience under the nearest listed value.

TABLE II

*Increases of Urea Quotient Observed in All Experiments with 10 Mg. per 100 Ml. of Ornithine Hydrochloride or Equivalent Concentration of Citrulline*

	Ornithine	Citrulline
Individual experiments	3.01 3.20 3.27 3.28 3.60 4.01 4.23 4.28 4.41 4.95 5.70	2.59 2.67 3.00 3.44 3.45 3.50 3.52 3.67 3.72 3.73 3.92 4.23 4.62 4.66
Mean.....	4.01	3.62
Parallel experiments	3.42 4.21 4.39 5.07 5.25 5.27 5.88 6.08	3.19 3.46 3.30 3.67 3.63 3.48 4.37 4.23
Mean.....	4.95	3.67
Mean of all experiments.....	4.38	3.64
Standard deviation of mean.....	$\pm 0.937$	$\pm 0.544$

Concentrations arranged in the same horizontal line are, it may be noted, molecularly equal. Detailed data concerning some of the experiments included in the summary may be seen in later tables.

Table II serves to exhibit the great variability of the results obtained at any one concentration of either catalyst, and to emphasize the impossibility of drawing valid conclusions from a comparison of single determina-

tions with each. It is true that in the *parallel* experiments of Table II the ornithine result is invariably the higher of the two, but at no other concentration has even this measure of consistency been observed. For this reason we have entered in the summary (Table III) not only the mean for each group, but the number of observations upon which it is based and its calculated standard deviation. We have further indicated those concentration levels (three only in number) at which, according to the *t* test of Fisher (18), the mean result for citrulline differs significantly from the mean for ornithine.

TABLE III

*Effects upon Urea Production of Increasing Concentrations of Ornithine, Citrulline, and Arginine; Summary of All Results*

NH<sub>4</sub> 0.015 per cent; Na lactate 0.2 per cent; time, 2 hours.

Ornithine HCl				Citrulline				Arginine HCl		
Mg. per 100 ml.	No. of observations	Mean increase of urea quotient	Standard deviation of mean	Mg. per 100 ml.	No. of observations	Mean increase of urea quotient	Standard deviation of mean	Mg. per 100 ml.	No. of observations	Mean increase of urea quotient
0.5	13	1.10	±0.57	0.52	14	0.75	±0.57	0.62	1	0.66
2	14	1.85	±0.71	2.08	19	1.34	±0.73	2.50	2	2.35
5	17	3.37	±0.75	5.2	23	2.38*	±0.57	6.24	4	5.00
10	20	4.38	±0.94	10.4	22	3.64*	±0.54	12.5	2	7.61
20	17	4.86	±1.06	20.8	15	4.75	±1.09	25.0	2	9.76
50	11	4.82	±1.20	52.0	6	4.80	±1.29	62.4	2	16.6
100	12	4.91	±1.48	104	12	5.14	±0.81	125	2	23.4
200	7	3.08	±1.16	208	3	4.33	±0.42			
300	1	2.92		312	1	3.58				
400	6	1.83	±0.94	416	2	3.03	±1.02			
500	8	1.82	±2.01	520	8	2.31	±1.25			
600	5	-0.19	±0.69	624	4	1.05*	±0.50			
1000	2	2.03	±0.04	1040	2	1.59	±1.08			

\* Significantly different from the corresponding ornithine result.

The data of Table III afford ample confirmation, if that were needed, of the original observations of Krebs and Henseleit upon the stimulation of urea production by citrulline as well as by ornithine. They provide, further, a larger material than has hitherto been available for comparing the relative efficiency of the two substances and the effects of varying concentrations of either. It is seen that in each case the effect increases regularly with concentration up to a level of 20 (in the case of ornithine perhaps only up to 10) mg. per 100 ml., that from 20 to 100 it remains constant, and that at still higher levels it falls off again, occasionally, with ornithine, reaching negative values. It may be said that, as far as ornithine

is concerned, the relations observed are in complete harmony with such relevant data as are to be found in the paper of Krebs and Henseleit (1). Comparable data for citrulline do not exist.

The catalytic nature of the ornithine effect was inferred by Krebs and Henseleit (1) from these two observations: (a) that the rise in urea production is not accompanied by any corresponding consumption of ornithine, and (b) that a small quantity of ornithine may promote the synthesis of several times its molecular equivalent of urea. In the present series of experiments the action of citrulline has been found to present the same two characteristics. Whenever a citrulline determination has been made at the end of a 2 hour experiment, it has yielded, within the experimental error, the amount which had been added at the beginning. As for the second point, it might be deduced from the mean values of urea quotient at low concentrations of citrulline, as given in Table III, but it is more

TABLE IV  
*Catalytic Effect of Citrulline*

Rat 23; volume of medium, 3.5 ml.; time, 2 hours.

Citrulline		Liver slices, dry weight	Extra CO: from urea	Urea increase	$\frac{\text{Moles urea}}{\text{Moles citrulline}}$
Concentration	In 3.5 ml.				
mg. per 100 ml.	micromoles	mg.	c.mm.	micromoles	
0.52	0.104	7.57	18.7	0.81	8.1
2.08	0.416	6.96	34.9	1.57	3.8
5.2	1.04	6.71	40.1	1.80	1.7
10.4	2.08	7.01	61.5	2.77	1.3

directly exhibited in a typical single experiment, such as that of which the data (with relevant calculations) are recorded in Table IV. On such evidence we conclude that citrulline exerts the same kind of catalytic effect as ornithine.

If, returning to Table III, we compare equivalent concentrations of ornithine and citrulline, it will be seen that in the region of increasing quotients the effect of the first appears to be greater than that of the second, and that at two levels (5 and 10 mg. per 100 ml.) the difference shown is statistically significant. At high concentrations this relation is reversed, although only at 600 mg. can the difference between means be said to have significance. Over the middle region of maximal action the effect of citrulline is indistinguishable from that of ornithine. In previous comparisons of this sort as reported by Krebs and Henseleit (1), Bach (4), and Isawa, Togo, and Kawabu (5), the effect of citrulline has appeared to be greater than that of ornithine. Details of Isawa's measurements are

not accessible. Those of Krebs and Henselcit, two in number, were made at an amino acid concentration of 200 mg. per cent, and are therefore in agreement with our own observations at that level. Bach reports but one experiment, in which, moreover, the respiratory substrate was not lactate, but ketoglutarate.

If, as the Krebs theory postulates, citrulline is a necessary intermediate in the production of urea from ornithine, it is evident that the stimulating effect of the latter cannot exceed that of an equivalent concentration of the first. One is forced therefore to consider the possible significance of the fact that at two concentration levels (5 and 10 mg. per 100 ml.) ornithine does apparently give a higher quotient than citrulline. This might mean that the Krebs cycle is not the only path along which ornithine is here giving rise to urea—that some alternative mechanism, such as that suggested by Bach (4), is simultaneously in operation. If this were so, one would hardly expect the difference to disappear, as it does, at higher concentrations. We prefer therefore to believe that the discrepancy is only apparent, and that it is to be attributed to factors which we have not yet learned either to recognize or to control. It has to be remembered that the *effective* concentrations of catalyst are those within the liver cell, and that these are not necessarily the same as the concentrations in the surrounding medium. The attainment of a given intracellular concentration may conceivably require a greater "head" of citrulline than of ornithine. In any case we consider the differences, such as they are, evident at low concentrations to be less significant than the identity of effect found over that whole broad span of concentrations (20 to 100 mg. per 100 ml.), in which the urea-producing mechanisms are working in the presence of an excess of catalyst and at the peak of their capacity. If ornithine and citrulline were involved in independent mechanisms, this identity would form a surprising coincidence. In the cycle of Krebs it is just what one would expect, provided only that, under the given conditions, reaction (II) is not more rapid than reaction (I). This proviso, it will presently be shown, is fulfilled, (II) being actually the slowest of the three reactions. We regard our results, then, as evidence that all of the extra urea observed in these experiments originated through the ornithine cycle.

As further evidence that the actions of ornithine and citrulline are parts of a single mechanism we may add that the combined effect of both never exceeds the maximal effect of one alone. Thus in one experiment (Rat 126) with 12 mg. per cent of each catalyst, and in three (Rats 127, 128, 129) with 60 mg. per cent of each, the increases of urea quotient observed were, respectively, 5.23, 4.71, 4.92, and 5.12. These are just such increases as occur with an optimal concentration of either catalyst by itself. Had each been operating upon a separate system, it might have been expected

that their effects would be additive; in that case the quotients would have reached much higher values.

The reason for the fall in urea production, which sets in at concentrations over 100 mg. per cent, is uncertain. We have considered the possibility that it might be due to the coming into effect of the well known inhibiting action of ornithine (or citrulline) upon the enzyme arginase, with a consequent blocking of the cycle specifically at reaction (III). Two facts appear to negative so simple an explanation of the phenomenon: (1) we have failed to detect any such accumulation of arginine as would necessarily result, and (2) the drop in urea production, whether with ornithine or citrulline, is accompanied (see Table VIII) by a drop in the consumption of ammonia, indicating a retardation of reactions (I) and (II). Amino acid concentrations of 0.2 to 1 per cent are of course quite unphysiological, and cannot be without effect upon osmotic and other relations of the liver cells. It seems likely that they produce a general depression of functional activity, of which the diminished production of urea is but one expression. With ornithine this effect was sometimes great enough to depress the urea quotient below its basal level (see in Table III, the mean value for 600 mg. per cent and, also, in Table VI, the individual values for Rats 92, 118, and 119).

The urea quotients shown in Table III for *arginine* rise to levels much higher than the peak attained with ornithine, and, as far as they go, show no indication of having an upper limit of their own. It seems evident then, even without closer analysis, that the rate of arginine hydrolysis (reaction (III)) cannot be a limiting factor in the speed of urea production. It is to be presumed, however, that under the existing circumstances the quotients observed reflect not only the direct production of urea from arginine by the liver arginase but, in summation therewith, the synthesis of urea through the action, within the Krebs cycle, of the ornithine simultaneously set free. In order to ascertain how far this presumption could be substantiated we calculated, for each individual experiment with arginine, the urea quotient which would have been obtained had the arginine been completely hydrolyzed and had there been no other source of urea. The results are given in the fourth column of Table V, where they may be compared with the mean increases of quotient actually found (third column). The comparison shows that at the lower concentrations of arginine (up to 25 mg. per 100 ml.) the extra urea produced is far from being accounted for by hydrolysis alone. The excess of the "found" over the "hydrolysis" quotient, entered in Table V as "surplus" quotient, we interpret as reflecting the catalytic effect upon urea synthesis of the ornithine directly derived from the added arginine. It can hardly be a coincidence that up to an arginine concentration of 12.5 mg. per 100 ml.

the surplus quotients calculated are not significantly smaller than those yielded by equivalent concentrations of originally uncombined ornithine (see Table III). We take this to mean that arginine, in concentrations not exceeding the limit indicated, is hydrolyzed by the liver slices so rapidly and so completely that its addition is equivalent, in effect upon urea synthesis, to that of so much free ornithine. With greater concentrations of arginine complete hydrolysis, it is evident, is not accomplished within the period of the experiment. The stimulating effect of "nascent" ornithine is accordingly either partially (as at the 25 mg. level) or entirely masked. It may be presumed not only that it still exists, but that it continues to increase up to, yet not beyond, the already determined maximum represented by a mean quotient of about 5. If this be granted, it will

TABLE V  
*Analysis of Effect of Arginine on Urea Production*

Concentration of arginine HCl	Corresponding concentration of ornithine HCl	Urea quotients		
		Mean increase above basal, as found (Table III)	Maximum possible by hydrolysis of arginine	Mean surplus by synthesis (ornithine cycle)
<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>			
0.625	0.5	0.66	0.20	0.46
2.50	2.0	2.35	0.95	1.40
6.25	5.0	5.00	2.14	2.86
12.5	10.0	7.61	4.26	3.35
25.0	20.0	9.76	7.70	(2.06)
62.5	50.0	16.6	18.6	
125.0	100.0	23.4	37.3	

appear that at the highest arginine concentration tested, where the quotient rose to 23.4 above basal, urea was being produced by direct hydrolysis several times as fast as by synthesis. Evidently, then, the liver is capable of hydrolyzing arginine much more rapidly than the ornithine cycle, working at the limit of its efficiency, can ever supply it.

*Production and Accumulation of Citrulline during Ornithine-Catalyzed  
• Synthesis of Urea<sup>1</sup>*

Table VI presents the record of those experiments, in which, using lactate as respiratory substrate and ornithine as catalyst, we determined not only the production of urea, but also that of citrulline. The number of rats employed in this series was twenty-two. The ornithine was added to the medium in concentrations ranging from 7.5 to 1000 mg. of the hydro-

<sup>1</sup> A preliminary account of the work described in this section was presented to the Royal Society of Canada in May, 1941 (19).



TABLE VI

*Accumulation of Citrulline during Urea Synthesis*NH<sub>3</sub> 0.015 per cent; Na lactate 0.2 per cent.

Rat No.	Ornithine HCl	Volume of medium	Dry liver tissue	Duration of experiment	Citrulline found	Citrulline quotient	Urea quotient	Coefficient of citrulline accumulation
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	mg. per 100 ml.	ml.	mg.	hrs.	mg. per 100 ml.			
82	0	3.5	10.5	2			5.67	
	50	3.5	10.2	2	2.0	0.44	11.1	0.038
	75	3.5	10.6	2	2.7	0.57	11.0	0.049
	100	3.5	10.2	2	3.6	0.79	11.2	0.066
85	0	3.5	13.6	3			4.44	
	20	3.5	14.0	3	1.3	0.13	7.32	0.017
	60	3.5	14.7	3	3.2	0.33	7.70	0.041
	100	3.5	13.2	3	3.9	0.41	8.51	0.049
87	0	3.5	12.2	3			4.74	
	75	3.5	12.3	3	2.2	0.27	9.62	0.027
89	0	3.5	11.5	2½			5.74	
	7.5	3.5	12.9	2½	0.3	0.016	9.01	0.005
	15	3.5	12.7	2½	0.6	0.094	8.94	0.010
	30	3.5	11.5	2½	1.2	0.21	10.0	0.021
	60	3.5	11.6	2½	2.6	0.44	9.68	0.043
90	0	3.5	17.4	2½			4.19	
	50	3.5	18.2	2½	1.5	0.17	7.62	0.022
	100	3.5	16.0	2½	3.0	0.37	8.33	0.043
	200	3.5	17.2	2½	4.7	0.55	8.01	0.064
	500	3.5	16.4	2½	13.0	1.58	5.33	0.23
91	0	3.5	12.2	2			5.65	
	100	3.5	12.0	2	3.0	0.56	10.6	0.050
	200	3.5	11.2	2	4.5	0.90	10.9	0.076
	400	3.5	12.7	2	6.8	1.20	8.79	0.12
	600	3.5	12.5	2	11.0	1.98	6.06	0.25
92	0	3.5	10.5	2			6.57	
	600	3.5	12.4	2	16.6	2.99	5.50	0.35
93	0	3.5	12.7	2			5.90	
	300	3.5	13.8	2	7.3	1.18	8.82	0.12
	600	3.5	13.8	2	13.0	2.11	6.33	0.25
94	0	3.5	10.7	2			5.16	
	75	3.5	10.3	2	4.1	0.89	12.1	0.069
	150	3.5	11.9	2	7.8	1.47	9.89	0.13
	450	3.5	11.4	2	18.7	3.67	7.23	0.34
95	0	3.5	11.4	2			4.97	
	500	3.5	11.0	2	11.9	2.42	8.45	0.22
	1000	3.5	10.6	2	14.5	3.07	6.98	0.31
97	0	3.5	7.60	2			5.38	
	100	3.5	7.46	2	2.6	0.78	12.2	0.060
	500	3.5	8.44	2	6.1	1.62	10.7	0.13
	1000	3.5	8.97	2	10.1	2.52	7.44	0.25

• TABLE VI—*Concluded*

Rat No.	Ornithine HCl	Volume of medium	Dry liver tissue	Duration of experiment	Citrulline found	Citrulline quotient	Urea quotient	Coefficient of citrulline accumulation
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	<i>mg. per 100 ml.</i>	<i>ml.</i>	<i>mg.</i>	<i>hrs.</i>	<i>mg. per 100 ml.</i>			
100	0	3.5	8.36	1			5.52	
	200	3.5	8.21	$\frac{1}{2}$	0.6	0.65	8.01	0.075
	200	3.5	8.31	1	1.1	0.59	8.89	0.062
	200	3.5	7.88	$1\frac{1}{2}$	2.8	1.06	10.1	0.095
	200	3.5	8.23	2	3.9	1.06	10.0	0.096
101	0	4.5	10.7	2			5.27	
	17	4.5	10.5	2	0.6	0.16	10.6	0.015
	75	4.5	11.0	2	2.7	0.71	10.4	0.064
	380	4.5	11.5	2	9.3	2.33	7.13	0.25
	380	4.5	11.2	2	9.3	2.39	7.41	0.24
107	23	4.5	10.1	2	0.3	0.086	11.2	0.008
	230	4.5	10.6	2	5.3	1.44	8.57	0.14
108	0	4.5	11.1	2			5.44	
	20	4.5	10.1	2	0.9	0.25	10.8	0.023
	200	4.5	12.1	2	7.4	1.76	8.11	0.18
	520	4.5	12.8	2	14.0	3.15	5.90	0.35
109	433	4.5	15.0	2	11.8	2.26	6.54	0.26
110	0	4.5	11.3	2			4.91	
	26	4.5	11.6	2	0.65	0.16	9.85	0.016
	520	4.5	12.3	2	12.3	2.88	7.39	0.28
111	0	4.5	13.3	2			4.63	
	433	4.5	13.8	2	6.6	1.38	7.27	0.16
117	0	4.5	11.1	$2\frac{1}{2}$			5.60	
	17	4.5	11.3	$2\frac{1}{2}$	0.6	0.14	9.68	0.014
	200	4.5	12.4	$2\frac{1}{2}$	6.1	1.26	7.35	0.15
	400	4.5	11.8	$2\frac{1}{2}$	8.2	1.78	6.32	0.22
	600	4.5	11.0	$2\frac{1}{2}$	10.4	2.42	5.65	0.30
118	0	4.5	11.0	$2\frac{1}{2}$			6.54	
	29	4.5	10.7	$2\frac{1}{2}$	0.4	0.096	10.5	0.009
	200	4.5	10.7	$2\frac{1}{2}$	3.0	0.72	9.06	0.074
	400	4.5	10.7	$2\frac{1}{2}$	6.5	1.56	7.83	0.17
	600	4.5	11.1	$2\frac{1}{2}$	10.9	2.51	5.77	0.30
119	0	4.5	13.3	$2\frac{1}{2}$			5.79	
	100	4.5	13.8	$2\frac{1}{2}$	5.3	0.98	8.78	0.10
	500	4.5	13.7	$2\frac{1}{2}$	21.0	3.93	4.36	0.47
	500	4.5	13.8	$2\frac{1}{2}$	18.1	3.36	4.28	0.44
156	0	4.5	9.32	2			4.57	
	10	4.5	10.5	2	0.16	0.044	9.46	0.005
	20	4.5	10.2	2	0.38	0.11	9.29	0.012
	30	4.5	10.2	2	0.63	0.18	9.47	0.019
	40	4.5	9.13	2	0.8	0.25	10.5	0.023
	80	4.5	9.24	2	1.3	0.40	11.5	0.034
	160	4.5	10.3	2	2.7	0.75	11.3	0.062

chloride per 100 ml. The basal performance of each liver (two excepted) was determined in a parallel experiment without added ornithine. In Table VI the observed gross rates of urea production are presented in the usual way as urea quotients (Column 8). The result of each citrulline determination is given in two ways, first, in Column 6, as the concentration actually found in the medium at the end of each experiment and, secondly, in Column 7, as a "citrulline quotient." The latter has been calculated as

$$Q_{\text{citrulline}} = \frac{\text{mg. citrulline in volume of medium}}{\text{mg. dry tissue} \times \text{hrs.}} \times \frac{22,400}{175}$$

and represents, in terms of the Krebs theory, the volume of (gaseous) ammonia (in c.mm. per mg. of dry tissue per hour) which, after uniting with ornithine in reaction (I), has failed to enter the later stages of the cycle. It is therefore directly comparable with the urea quotient which, although originally defined in terms of  $\text{CO}_2$ , must, if the theory is correct, be a measure also of (a) ammonia taken up in reaction (I) and carried on through subsequent stages to appear in urea or, equal therewith, (b) ammonia taken up in reaction (II). The sum of the two quotients will give the total amount of ammonia, per mg. and hour, entering into the cycle with reaction (I). The fraction of this total which fails to go further may be called the "coefficient of citrulline accumulation," or simply the "citrulline coefficient." Calculated as

$$\frac{\text{Citrulline quotient}}{\text{Citrulline quotient} + \text{urea quotient}}$$

it has been entered in Column 9 of Table VI.

The prime result of the experiments is the demonstration that, when liver slices from fasted animals are suspended in a medium containing ammonia, sodium lactate, and ornithine, the customary acceleration of urea synthesis is accompanied by an accumulation of appreciable, and under favorable conditions substantial, quantities of citrulline. No certain indications of the presence of citrulline have ever been obtained in the absence of added ornithine, but in the presence of as little as 0.0075 per cent of the hydrochloride its concentration reaches measurable dimensions within 2 hours (Rat 89), and it seems a fair inference that some slight accumulation occurs at levels even lower. The data, it is to be noted, present no exceptions; whenever ornithine (within the range of concentrations tested) was present, citrulline made its appearance. Up to at least 2 hours the production of citrulline, like that of urea, is progressive with time (Rat 100). Both, likewise, are functions of the intact liver cell.

Thus in the presence of 75 mg. per cent of ornithine liver slices from Rat 87 produced in 3 hours a citrulline concentration of 2.2 mg. per 100 ml. Under the same conditions an equal weight of macerated tissue from the same liver produced no detectable quantity.

It will hardly be doubted that the citrulline found in these experiments was synthesized from the added ornithine, but, if any evidence beyond their chemical relationship be required, it will be found in the correspondence exhibited between the ornithine concentration of the medium and the quantity of citrulline produced. It is true that at any one ornithine level the citrulline quotients of different livers vary as widely as the coincident increases of urea quotient. Thus at 200 mg. per cent of ornithine we find citrulline quotients varying from 0.55 (Rat 90) to 1.76 (Rat 108). But for any individual liver each rise in ornithine concentration is accompanied invariably by an increased accumulation of citrulline. At the lower ornithine levels (up to about 100 mg. per cent) ornithine concentration and citrulline accumulation are, in fact, almost exactly proportional to one another (see Rats 89, 90, 94, 101). At higher levels proportionality commonly fails (Rat 118 presenting here a striking exception), but citrulline values still continue to rise. The highest concentrations actually attained in individual experiments of the series were 18.7 (Rat 94) and 19.6 (average, Rat 119) mg. per cent, the citrulline quotients corresponding being 3.67 and 3.65. The ornithine concentration, at which these rather remarkable accumulations of citrulline were observed, was 450 to 500 mg. per 100 ml. It is not to be inferred that that concentration is a limiting one. The results with Rats 94 and 119 are somewhat exceptional; Rats 95 and 97 show that an increase of ornithine from 0.5 to 1 per cent is still accompanied by a rise in the rate of citrulline accumulation. The data suggest in fact that with increasing ornithine concentration the citrulline quotient will rise indefinitely. Its behavior is therefore altogether different from that of the quotient for either extra or total urea.

The inferences thus drawn from individual experiments are supported by a comparison of the averages, which, along with certain other calculated data, are recorded in Table VII. These averages are obtained by grouping, as indicated, all results falling within each of certain reasonably narrow ranges of ornithine concentration. It will be evident that the citrulline data thus averaged, whether as concentrations, quotients, or coefficients, lie on or near a curve, the initial part of which, up to an ornithine concentration of about 100 mg. per 100 ml., is a straight line. The curve for quotient continues to rise, although at a diminishing rate, to the end. The coefficient appears to reach a maximum at 0.5 to 0.6 per cent of ornithine, but this may be merely because the two observations made at 1 per cent were too few to give a fully representative average. It will be

noted that at that level the urea quotient, as well as the sum of the two quotients, also falls out of line.

The results as a whole we take as demonstrating (a) that liver slices can convert ornithine into citrulline and (b) that such conversion is a constant accompaniment of the synthesis, through added ornithine, of urea. It has already been shown that citrulline stimulates the production of urea in essentially the same degree as ornithine. Taken in conjunction these findings fall little short of proof that citrulline lies on a path between ornithine and urea. To that extent they furnish direct and cogent evidence in support of the hypothesis of Krebs and Henseleit.

Taking that hypothesis, accordingly, as firmly established, we regard the accumulation of citrulline in our experiments as proof that it was being converted into arginine less rapidly than it was produced from ornithine.

TABLE VII  
*Citrulline Accumulation; Averaged Data*

Concentration of ornithine HCl, mg. per 100 ml.....	7.5-15 (11)*	17-30 (23)	40-80 (66)	100-160 (114)	200-250 (205)	350-450 (415)	500-600 (550)	1000
No. of examples .....	3	10	10	8	7	7	11	2
Citrulline found, average, mg. per 100 ml. ....	0.35	0.70	2.3	4.0	5.0	9.7	12.6	12.3
Citrulline quotient, average ..	0.061	0.15	0.45	0.77	1.10	2.03	2.48	2.80
Urea quotient, average.....	9.14	9.87	10.12	10.10	8.86	7.32	6.49	7.21
Sum of average quotients....	9.20	10.02	10.57	10.87	9.96	9.35	8.97	10.01
Citrulline coefficient, average.	0.007	0.015	0.041	0.070	0.11	0.22	0.28	0.28

\* The figures in parentheses are averages.

The question presents itself, whether this may not have been a quite unphysiological state of affairs, due to a more or less specific inhibitory effect of unusual concentrations of ornithine upon reaction (II). The data of Table VII appear to show that a suspicion of this sort is partly, but only partly, justified. It will be noted there that the sum of the urea and citrulline quotients (which, as already pointed out, reflects the rate of reaction (I)) continues to rise up to an ornithine concentration of about 100 mg. per cent, and that its subsequent falling off is rather slow. The urea quotient (reflecting similarly the rate of reaction (II)) falls off much more rapidly, nearly twice as rapidly, if we neglect the aberrant values at 1 per cent of ornithine. It does appear therefore that the depressant action of high concentrations of ornithine affects chiefly the step from citrulline to arginine. On the other hand citrulline accumulation is by no means limited to that upper range of ornithine concentrations within which depression becomes evident. It is already conspicuous while urea synthesis is

at its maximum, and is at least perceptible even earlier. We conclude that with ornithine concentrations up to the optimal the factor limiting the rate of urea production (that is, the over-all efficiency of the whole cycle) is the speed with which citrulline can be transformed into arginine (reaction (II)). This conclusion, it has already been pointed out, is in consonance with the equal efficacy of ornithine and citrulline as catalysts.

It is probable that in the intact organism urea production seldom reaches such heights that the limiting factor actually comes into play. Only if and when it does, would citrulline be expected to accumulate *in vivo*. Conceivably it is as a provision against just such a contingency that the kidney, as Borsook and Dubnoff (12) have shown, possesses the power to convert citrulline into arginine. By that mechanism citrulline would be returned to the cycle at a point where the functional capacity of the latter is practically unlimited.

The calculation of "citrulline coefficients" indicates (Table VII) that at ornithine concentrations of 17 to 30 mg. per 100 ml. only about 1.5 per cent of the ammonia utilized in reaction (I) fails to complete the entire cycle, whereas at 0.5 to 1 per cent of ornithine the proportion retained in accumulating citrulline rises on the average to 28 per cent. In individual experiments the proportion may be even higher, 35 per cent in Rats 92 and 108, and nearly 50 per cent in Rat 119 (see Table VI). In the last instance the medium actually contained, at the end of the experiment, about  $2\frac{1}{2}$  times as much citrulline (by weight) as urea.

#### *Relation between Urea Production and Ammonia Consumption*

A number of rats were devoted to experiments in which we attempted to determine not only the amount of urea (and in some cases of citrulline) produced, but also the quantity of  $\text{NH}_3$  which disappeared from the medium. Some of the results had to be rejected on account of the doubtful accuracy of the earlier ammonia determinations (see "Methods"). The remainder, constituting a group of, as we believe, trustworthy observations upon ten separate livers, is presented in Tables VIII and IX. In each experiment there recorded the medium contained as usual 0.2 per cent sodium lactate and about 0.015 per cent  $\text{NH}_3$ , with which there were incorporated varying proportions, as indicated in the tables, sometimes of ornithine, sometimes of citrulline. The volume used, always 4.5 ml., contained (as separately determined for each experiment) from 543 to 568  $\gamma$  of ammonia nitrogen. The figures given for  $\text{NH}_3\text{-N}$  consumed represent, of course, in each instance the difference between the initial ammonia content of the medium and the ammonia found at the end of the experimental period. This period was usually 2 hours, but occasionally (Rats 117, 118, 119)  $2\frac{1}{4}$  hours. The dry weight of liver tissue employed varied

TABLE VIII

*Influence of Ornithine and Citrulline upon Relation between Ammonia Consumed and Urea Produced*

Rat No.	Concentration of catalyst		Ammonia N consumed (A)	Urea N produced (B)	Ratio $\frac{A}{B}$		
	Ornithine HCl	Citrulline			Without catalyst	With ornithine	With citrulline
	mg. per 100 ml.	mg. per 100 ml.	$\gamma$	$\gamma$			
101	17		307	278		1.10	
		18	293	273			1.07
107	23		306	283		1.08	
		24	269	289			0.93
		240	267	267			1.00
108			166	151	1.10		
	20		296	273		1.08	
		21	301	286			1.06
		210	297	287			1.03
110	26		326	288		1.13	
		27	313	279			1.12
117			203	175	1.16		
	17		332	308		1.08	
		18	300	280			1.07
	600		223	174		1.28	
		500	247	249			0.99
118			240	202	1.19		
	29		348	318		1.09	
		28	340	317			1.07
	600		222	180		1.23	
		625	233	235			0.99
119			244	217	1.12		
	100		352	342		1.03	
		50	355	362			0.98
	500		270	168		1.61	
	500		252	166		1.52	
		500	220	234			0.94
78			258	231	1.12		
		100	418	413			1.01
79			262	224	1.17		
		100	405	416			0.97
116		100	313	297			1.05
		500	258	269			0.96
Mean values of A:B, all observations					1.14	1.20	1.02
" " " " for catalyst concentra- tions below 30 mg. per 100 ml.						1.09	1.05
Mean values of A:B, for catalyst concentra- tions of 500-600 mg. per 100 ml.						1.41	0.97

for different rats between 10 and 17 mg., but for all experiments with any one rat was made as nearly as possible the same. In most cases the basal performance of the liver was measured in a parallel experiment without added catalyst.

Table VIII shows that (a) under basal conditions and (b) with citrulline as catalyst the ratio of ammonia nitrogen used up to urea nitrogen formed is reasonably constant, never differing by more than 10 per cent from a mean value of 1.14 in the first case, or 1.02 in the second. Bach's (4) finding of a ratio of approximately 1 for citrulline is therefore fully confirmed. It is further evident that this ratio is independent of the concentration of citrulline, being essentially the same with 200 or 600 mg. per 100 ml. as with 20. With ornithine the ratio is found to be more variable. It ranges from 1.03 to 1.61, and its mean value in our series (1.20) is definitely greater than the mean with citrulline. There is to be noted, however, an evident association of the higher ratios with high concentrations of ornithine. Neglecting for the present a probable explanation of this association, we may remark now that for concentrations of ornithine not exceeding 30 mg. per 100 ml. the ratio is again approximately 1 (mean value found = 1.09). Within the limitation stated the ratios for citrulline and ornithine catalysis are therefore essentially the same. This identity of effect is shown not only by the calculated means, but also (and even better) by all but one (Rat 107) of the pairs of values (braced in Table VIII) obtained when ornithine and citrulline, in the same moderate concentration, were compared in action upon a single liver.

A nitrogen ratio of 1 means that for each molecule of urea produced 2 molecules of  $\text{NH}_3$  have been used up. It is therefore the ratio to be expected from the continuous operation of the ornithine cycle, provided always that at no point in that cycle does ammonia become, so to speak, imprisoned in an accumulating intermediate. With this proviso it ought not to matter whether the cycle is regarded as starting from ornithine or from citrulline, or which of these substances is acting in any particular instance to increase the over-all performance of the cycle. The fact that the steps from citrulline to urea (reactions (II) and (III)), taken by themselves, involve a ratio of only 0.5 is hardly relevant. These steps lead to the production not only of urea, but simultaneously of ornithine, and this, according to the theory, must at once take up an additional quota of  $\text{NH}_3$ . It was by this consideration that Krebs and Henseleit (1) offered to explain the ratio of 0.69, instead of 0.5, found in the single experiment which they carried out with citrulline. One has only to push the argument to its logical conclusion to see that citrulline should give a ratio less than 1 only if ornithine fails to react with ammonia as rapidly as it is produced, through arginine, from citrulline; in other words, only if reaction (I) takes place



more slowly than reaction (II). Under such conditions one would expect ornithine to accumulate and the concentration of citrulline to fall. Now Bach (4) has shown that during the synthesis of urea, as stimulated by citrulline, there occurs no formation of ornithine, and we ourselves, under similar circumstances, have repeatedly failed to find any change in the concentration of the citrulline. We have, moreover, already given cogent grounds for believing that reaction (I) is not less, but more, rapid than reaction (II). A ratio of 1 for citrulline is therefore not inconsistent with the hypothesis of Krebs and Henseleit. On the contrary, viewed in the light of the relative slowness of the citrulline-to-arginine transformation,

TABLE IX

*Citrulline Accumulation and Ammonia-Urea Ratio with Ornithine As Catalyst*

Rat No.	Ornithine HCl	Ammonia N consumed (A)	Urea N produced (B)	Citrulline N (1 atom) (C)	Ratio $\frac{A}{B}$	Ratio $\frac{A}{B+C}$
	mg. per 100 ml.	γ	γ	γ		
117	17	332	308	2	1.08	1.07
	200	305	256	22	1.19	1.10
	400	252	210	30	1.20	1.05
	600	223	174	38	1.28	1.05
118	29	348	318	1.5	1.09	1.09
	200	292	272	11	1.07	1.03
	400	265	235	24	1.13	1.02
	600	222	180	39	1.23	1.01
119	100	352	342	19	1.03	0.98
	500	270	168	76	1.61	1.11
	500	252	166	65	1.52	1.09
Mean.....						1.05

it affords to that theory additional support. The relatively low ratio observed by Krebs and Henseleit we feel bound to regard as fortuitous.

We may revert now to the high ratios (mean value = 1.41) found in the presence of excessive concentrations of ornithine. A ratio greater than 1 means of course that  $\text{NH}_3$  has entered, and has remained in, other combinations than urea. Considerable accumulations of one such other combination, citrulline, have already been shown to appear under exactly those circumstances which induce a high ratio of ammonia to urea. It becomes pertinent therefore to inquire how much of the  $\text{NH}_3$  not accounted for as urea can be located in citrulline. An answer to this question was provided by the experiments with Rats 117, 118, and 119, in which determinations of citrulline were combined with those of ammonia and urea. The citrulline results have already been recorded in Table VI. In utilizing them in

the present connection it has been assumed, in accord with the theory, that 1 of the 3 atoms of nitrogen in citrulline has been derived from  $\text{NH}_3$ . Table IX presents the results obtained on this basis, and shows that, when the  $\text{NH}_3\text{-N}$  to urea N ratios are "corrected" by including in the denominator the  $\text{NH}_3$  bound up in citrulline, they are reduced to a practically uniform level never differing greatly from unity. The mean of all corrected values is 1.05. This signifies that on the average 95 per cent of the  $\text{NH}_3$  utilized during each experiment has been accounted for as urea and citrulline; i.e., by the operation of the ornithine cycle. In no single instance did more than 10 per cent appear to have followed some other path. These experiments furnish therefore yet another item supporting the hypothesis of Krebs and Henseleit.

#### DISCUSSION AND SUMMARY

Mechanisms of urea formation, alternative or supplementary to that of the ornithine cycle, have been proposed by Leuthardt (20) and by Bach (4). We have not neglected the opportunity of testing the reported results of these authors, but our experiments in that direction are as yet too few to justify definite conclusions. We refrain therefore for the present from discussing the question whether under any circumstances urea synthesis may proceed, in whole or in part, according to some other scheme than that of Krebs and Henseleit. The experiments we have here reported were made under one set of conditions only; namely, with lactate as respiratory substrate and with ammonia, in sufficient excess, as the sole added source of nitrogen. The results of these experiments we have interpreted as affording direct support to, or as entirely consistent with, the hypothesis of the ornithine cycle. Direct support is furnished by the demonstration (a) that citrulline exerts upon urea production the same kind of catalytic effect as ornithine, and (b) that when ornithine acts as the catalyst it does actually give rise to citrulline. The fact that production of urea under the action of ornithine is accompanied by an accumulation of citrulline, an accumulation perceptible even at suboptimal concentrations of the catalyst, is taken to show that the limiting factor in the operation of the cycle is the capacity of the liver tissue to convert citrulline into arginine. In full consonance with this idea, it has been argued, are the observations (a) that at optimal concentrations (20 to 100 mg. per 100 ml.) ornithine and citrulline exert, molecule for molecule, identical effects upon the rate of urea production, (b) that with citrulline, as with low concentrations of ornithine, the ratio of  $\text{NH}_3\text{-N}$  consumed to urea N produced is unity, and (c) that the larger ratios found with high concentrations of ornithine are completely accounted for by the coincident accumulation of citrulline. The only discrepant results are those which suggest

a greater effect of ornithine, as compared with citrulline, at concentrations below 20 mg. per 100 ml. Although this discrepancy remains for the present unexplained, we have given reason for doubting that it is of crucial importance. We conclude, accordingly, that under the conditions of our experiments all the phenomena of urea production (the one exception ignored) are immediately explicable in terms of the Krebs-Henseleit hypothesis, amplified only by the postulate that the transformation of citrulline into arginine is the slowest of the three reactions involved. The formulation of alternative schemes is therefore here superfluous.

Some comment is called for by the recent paper of Trowell (21). This paper describes perfusion experiments, performed upon rat livers, the outcome of which led Trowell to deny that citrulline showed, at low concentrations, any effect whatsoever or, at high concentrations, any effect of a catalytic nature. These and other results were interpreted as demonstrating that the ornithine cycle cannot account for the whole of the urea formed from ammonia, and that in its original form it is in fact untenable. Trowell's results with citrulline are in complete contrast with those reported in the present paper. The explanation of this contrast is not immediately apparent. Presumably it is to be found in some defect of the perfusion technique, which presents perhaps greater opportunity than the other for the occurrence of disturbing side reactions. In any case negative results obtained by its use can hardly be held to outweigh the clear cut positive results yielded by the tissue slice method. It may be added that in stating, as a weakness of the ornithine theory, that "there is no evidence that ornithine is ever converted into arginine" Trowell ignores the proof of such conversion afforded by the isotope work of Clutton, Schoenheimer, and Rittenberg (6).

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*Addendum (February 12, 1948)*—This paper was already in page proof when we received the December, 1942, issue of the *Biochemical Journal*, containing a communication by Krebs (22) on "Urea formation in mammalian liver." Among the experiments therein reported there are five in which the effects of ornithine and citrulline, in concentrations of from 50 to 180 mg. per 100 ml., are directly compared. In four of these the rates of urea formation were "virtually identical." This is, of course, in complete consonance with our own results. Referring to our earlier mention (17) of citrulline accumulation, Krebs deduces, as we had already done (19), that "here the conversion of citrulline into arginine is probably the limiting reaction." Krebs also presents data, similar to those in our Table IV, demonstrating the catalytic effect of citrulline.

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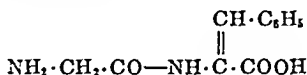
# PEPTIDES OF DEHYDROGENATED AMINO ACIDS

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Some 12 years ago, extracts of swine kidney were found to contain an enzyme, designated dehydrodipeptidase, that hydrolyzes the unsaturated peptide glycyldehydrophenylalanine



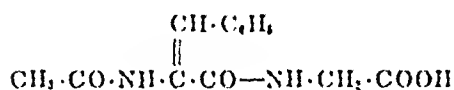
to yield glycine, phenylpyruvic acid, and ammonia (1, 2). The existence of this enzyme was taken as an indication that dehydrogenated peptides might occur in metabolism. In order to facilitate the further study of this possibility, it was necessary to synthesize a number of peptides containing dehydrogenated amino acids. The present paper is concerned with the description of such peptides and the methods employed in their preparation.

As has previously been shown, acylated peptides containing one dehydrogenated amino acid residue may be obtained by coupling in acetone-water solution the azlactone of an unsaturated amino acid with the sodium salt of a saturated amino acid. This reaction scheme has previously been employed for the synthesis of dehydrogenated peptides containing glycine, *l*-leucine, *l*-glutamic acid, *l*-tyrosine, *l*-proline, *l*-arginine, and *dl*-serine (3, 4).<sup>1</sup> Evidence of the good yields and high degree of purity of the products obtained by this procedure will be found in the experimental section; *e.g.*, pure acetyldehydrophenylalanylglycine (I) has now been obtained in 90 per cent yield.

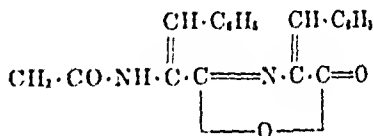
It has now been found that peptides with a glycine group at the carboxyl end, when treated with an aromatic aldehyde at 40° in the presence of acetic anhydride and sodium acetate, will yield the azlactones of unsaturated peptides. Thus, peptide (I) yielded the azlactone (II) of the doubly unsaturated peptide acetyldehydrophenylalanyldehydrophenylalanine (III). The latter peptide was obtained from the azlactone by alkaline hydrolysis.

<sup>1</sup> The coupling of saturated amino acid azlactones with amino acids in the presence of anhydrous pyridine has been studied by Carter, Handler, and Stevens (5).

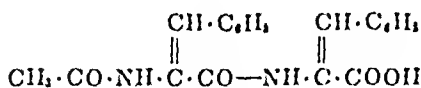
The use of *p*-hydroxybenzaldehyde in place of benzaldehyde makes accessible peptides containing the dehydrogenated tyrosine residuc.



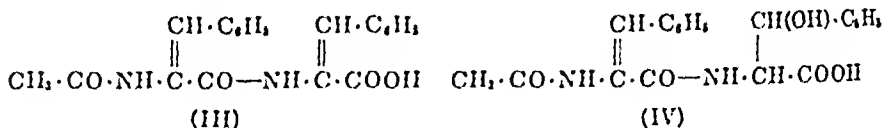
(I)



(II)

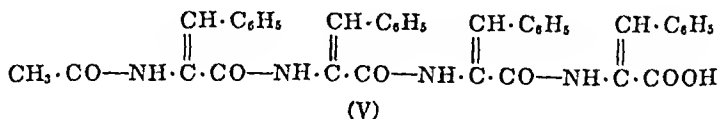


(III)



(IV)

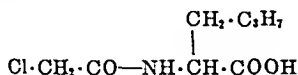
Azlaetone (II) was also obtained in a second manner. The azlaetone of  $\alpha$ -acetaminocinnamic acid (N-acetyldehydrophenylalanine) was coupled with *trans*-phenylserine (6) to yield peptide (IV). This intermediate, on treatment with acetic anhydride at room temperature in the presence of small amounts of anhydrous sodium acetate, forms the azlaetone (II). This transformation of peptide (IV) into azlaetone (II) in all probability does not consist merely of a splitting off of 2 water molecules; we assume that, at an intermediary stage, the  $\beta$ -hydroxyl of the phenylserine residue is acetylated and that subsequently, acetic acid is eliminated from the molecule.<sup>2</sup> There will be described in the experimental section several other peptides, each containing a terminal phenylserine residue, which, under the influence of acetic anhydride, were found to split off 2 molecules of water and to yield azlaetones. The azlaetone of the quadruply unsaturated peptide (V) was obtained by repeated applications of this procedure.



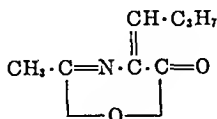
All the hitherto described reactions yield peptides of dehydrogenated aromatic amino acids. Peptides containing a dehydrogenated aliphatic amino acid were obtained in the following manner. It is known that chloroacetylated amino acids may be easily transformed into the azlaetones of dehydrogenated amino acids (8). Correspondingly, chloroacetylucine (VI) has now, by treatment with acetic anhydride, been converted into the

<sup>2</sup> A similar reaction sequence concerning a cyclic derivative of serine is described in (7).

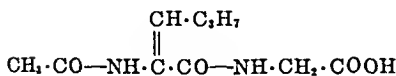
azlactone (VII) of acetyldehydroleucine. From this azlactone we prepared the peptide acetyldehydroleucylglycine (VIII), the azlactone (IX) of acetyldehydroleucyldehydrophenylalanine (X), and finally peptide (X) itself.



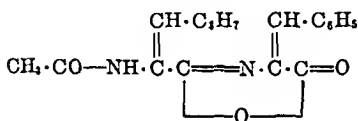
(VI)



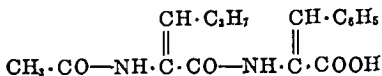
(VII)



(VIII)



(IX)



(X)

In the interest of brevity, the unsaturated peptides obtained by the aforementioned methods are not discussed in detail but only listed as follows:<sup>3</sup>

#### *Dipeptides with One Double Bond*

Acetyldehydrophenylalanylphenylserine  
 Benzoyldehydrophenylalanylglycine  
 Benzoyldehydrophenylalanylphenylserine  
 Acetyldehydroleucylglycine  
 Carbohenzoxyglycyldehydrophenylalanine<sup>4,5</sup>  
 Acetyldehydrophenylalanyl-*l*-alanine  
 Acetyldehydrophenylalanyl-*l*-phenylalanine  
 Acetyl-*dl*-phenylalanyldehydrophenylalanine<sup>4</sup>

#### *Dipeptides with Two Double Bonds*

Acetyldehydrophenylalanyldehydrophenylalanine<sup>4,5</sup>  
 Benzoyldehydrophenylalanyldehydrophenylalanine<sup>4,5</sup>  
 Acetyldehydrophenylalanyldehydrotyrosine<sup>4</sup>  
 Benzoyldehydrophenylalanyldehydrotyrosine<sup>4,5</sup>  
 Acetyldehydroleucyldehydrophenylalanine<sup>4</sup>

<sup>3</sup> The experimental section follows the sequence of this list as closely as possible.

<sup>4</sup> The azlactone of this compound is also described in the experimental section.

<sup>5</sup> The amide of this compound is also described in the experimental section.



*Tripeptides with One Double Bond*

Carbobenzoyglycyldehydrophenylalanyl-L-glutamic acid

Carbobenzoyglycyldehydrophenylalanylphenylserine

*Tripeptides with Two Double Bonds*

Acetylbis(dehydrophenylalanyl)glycine

Acetylbis(dehydrophenylalanyl)-L-alanine

Acetylbis(dehydrophenylalanyl)-L-leucine

Acetylbis(dehydrophenylalanyl)-L-phenylalanine

Acetylbis(dehydrophenylalanyl)-L-tyrosine

Acetylbis(dehydrophenylalanyl)-L-proline

Acetylbis(dehydrophenylalanyl)phenylserine

Acetylbis(dehydrophenylalanyl)-L-glutamic acid

Bis(acetyldehydrophenylalanyl)-L-cystine

*Tripeptide with Three Double Bonds*

Acetylbis(dehydrophenylalanyl)dehydrophenylalanine (azlactone only)

*Tetrapeptides with Three Double Bonds*

Acetyltris(dehydrophenylalanyl)-L-phenylalanine

Acetyltris(dehydrophenylalanyl)phenylserine

*Tetrapeptide with Four Double Bonds*

Acetyltris(dehydrophenylalanyl)dehydrophenylalanine (azlactone only)

*Pentapeptide with Four Double Bonds*

Bis(acetyldehydrophenylalanylddehydrophenylalanyl)-L-cystine

The peptides and azlactones containing one or several double bonds may be expected to exist in several stereoisomeric forms. The azlactone of acetyldehydrophenylalanylddehydrophenylalanine was indeed obtained in two forms of different color and melting point.

In Table I there are reported the molecular rotations in pyridine solution of the pyridine salts of a number of acetylated unsaturated peptides. The peptides compared have an optically active amino acid at the carboxyl end and one or more dehydrophenylalanine residues between the acetyl group and the optically active amino acid residue. The samples employed were analytically pure and exhibited upon recrystallization no appreciable change of rotation. While this behavior does not absolutely guarantee optical homogeneity, none of the peptides showed any indication of inhomogeneity.

It will be noted that the peptides of alanine, leucine, phenylalanine, and tyrosine containing a single dehydrophenylalanine residue show a positive rotation and that the corresponding peptides with two dehydrophenylalanine residues exhibit a rather high negative rotation. The peptides of

proline do not follow this general scheme. Acetyldehydrophenylalanyl-*L*-proline has a positive rotation not very much higher than that of the analogous alanine peptide; acetylbis(dehydrophenylalanyl)-*L*-proline also is dextrorotatory and thus differs greatly from the other doubly unsaturated peptides. The glutamic acid series deviates from the above general scheme in another manner. The acetyldehydrophenylalanyl peptide is described in the literature (3, 4) as having a rather small levorotation. The acetylbis-(dehydrophenylalanyl)-*L*-glutamic acid has now been found to show a considerably higher levorotation.

When cystine is subjected to the azlaetone procedure, both its amino groups are substituted. Two cystine peptides are reported in Table I, the one containing two acetyls and two dehydrophenylalanine residues per

TABLE I  
*Molecular Rotations of Several Dehydrogenated Peptides (at Temperatures Ranging from 28–53°)\**

<i>L</i> -Amino acid residue	Acetyldehydro- phenylalanyl derivative (1)	Acetylbisdehydro- phenylalanyl derivative (2)	Acetyltrisdehydro- phenylalanyl derivative (3)	$\Delta$ , (1) – (2)
Alanine.....	+19,200	–110,300		129,500
Leucine.....	+11,800	–113,700		125,500
Phenylalanine.....	+12,700	–85,500	–23,100	98,200
Tyrosine.....	+16,600	–70,700		87,300
Glutamic acid.....	–1,500	–87,500		86,000
Proline.....	+25,000	+22,200		2,800
Cystine.....	–11,800	–77,600		

\* The temperatures at which the rotations were determined may be found in the experimental section.

cystine residue and the other containing two acetyls and four dehydrophenylalanine residues. Both peptides are levorotatory.

A common characteristic of all the above peptides is the fact that in each series the transition from the acetyldehydrophenylalanyl peptide to the acetylbis(dehydrophenylalanyl) peptide results in a shift of the rotation towards the left.

However, it will be observed that no further shift towards the left occurs but, on the contrary, a considerable decrease of the levorotation when one proceeds from acetylbis(dehydrophenylalanyl)-*L*-phenylalanine to acetyltris(dehydrophenylalanyl)-*L*-phenylalanine.

The above data, preliminary though they are, already indicate clearly that the rotation of unsaturated peptides is a function of the structural nature of the active amino acid residue as well as of the number of un-

saturated residues present. The expectation may be expressed that a closer study of unsaturated, optically active peptides, which are now sufficiently accessible, will result in valuable contributions to our understanding of the interdependence of optical rotation and molecular structure in peptides.

Several unsaturated peptides were found to undergo easy transformation into cyclic anhydropeptides which are isomeric with azlactones. The synthesis of these anhydropeptides' will be reported in a later communication.

The authors wish to thank Mr. Stephen M. Nagy, who performed most of the analyses reported in the experimental section, and also Dr. Adalbert Elek, for their valuable assistance.

#### EXPERIMENTAL

*Acetyldehydrophenylalanylglycine*—The previously given directions (9) for the preparation of acetyldehydrophenylalanylglycine were modified as follows: To a suspension of 30 gm. of glycine in 400 cc. of acetone were added with stirring 400 cc. of *N* NaOH and, after several minutes, 75 gm. of acetyldehydrophenylalanine azlactone (acetaminocinnamic acid azlactone). After several hours 410 cc. of *N* HCl were added to the clear solution. 95 gm. of acetyldehydrophenylalanylglycine crystallized at 0°. These crystals were dissolved in aqueous potassium bicarbonate and reprecipitated by the addition of HCl. M.p., 194–195° (corrected).

*Acetyldehydrophenylalanylphenylserine*—To a suspension of 19 gm. of *trans*-phenylserine (6) in 300 cc. of acetone were added with stirring 100 cc. of *N* NaOH and 19 gm. of acetyldehydrophenylalanine azlactone. After 1 hour's stirring at 45°, the resulting clear solution was acidified at 0° by the addition of 102 cc. of *N* HCl and 28 to 30 gm. of the acetylated dipeptide crystallized. This was dissolved in a hot mixture of 600 cc. of ethanol and 180 cc. of water. The cooled solution gave on dilution with more water 27 to 29 gm. of colorless needles. M.p., 226–228° (with decomposition) (corrected).

$C_{20}H_{20}O_5N_2$ .	Calculated.	C 65.2, H 5.5, N 7.6
368.4	Found.	" 65.2, " 5.6, " 7.7

This peptide is somewhat soluble in hot aqueous acetone, but much less so in anhydrous acetone, benzene, chloroform, and water.

For the transformation into the azlactone of acetyldehydrophenylalanyldhydrophenylalanine, 20 gm. of acetyldehydrophenylalanylphenylserine were kept at 40° with 50 cc. of acetic anhydride and 2 gm. of anhydrous sodium acetate for 24 hours. After the excess acetic anhydride was

decomposed by ice water, the filtered azlactone was kept overnight with 100 cc. of acetone at 0°. Yield, 13.5 gm. The azlactone was dissolved in warm acetone and reprecipitated by the addition of water. M.p., 184–186° (corrected).

$C_{20}H_{16}O_4N_2$ .	Calculated.	C 72.3, H 4.8, N 8.4
332.3	Found.	" 72.1, " 5.1, " 8.5

*Benzoyldehydrophenylalanylglycine*—A solution of 30 gm. of glycine in 400 cc. of *N* NaOH and 125 cc. of acetone was stirred with 100 gm. of the azlactone of benzoyldehydrophenylalanine. After 2 to 3 hours, the addition of 402 cc. of *N* HCl precipitated the colorless rods of benzoyldehydrophenylalanylglycine. Yield, 120 gm. The benzoyl peptide was dissolved in acetone and crystallized on addition of water. M.p., 208–209° (with decomposition) (corrected).

$C_{18}H_{14}O_4N_2$ .	Calculated.	C 66.6, H 5.0, N 8.6
324.3	Found.	" 66.7, " 5.1, " 8.8

A compound of the same designation but a melting point of 165° has been obtained by Graenacher and Mahler (10) by coupling benzoyldehydrophenylalanine azlactone with glycine ethyl ester and subsequently saponifying the ester group with hot NaOH. By saponifying the ester at room temperature with *N* NaOH and with the addition of acetone, we obtained a benzoyldehydrophenylalanylglycine melting at 199–200° (with decomposition). A mixture with the preparation obtained from benzoyldehydrophenylalanine azlactone and glycine melted at 200–201°. No effort was made to decide whether the lower melting preparations were mixtures of stereoisomeric compounds.

*Benzoyldehydrophenylalanylphenylserine*—To a suspension of 36.2 gm. of *trans*-phenylserine in 560 cc. of acetone there were added with stirring 200 cc. of *N* NaOH and subsequently 50 gm. of benzoyldehydrophenylalanine azlactone and the temperature was raised to 55°. After 2 to 3 hours, the clear solution was acidified, at 0°, by the addition of 205 cc. of *N* HCl. Yield, 58.4 gm. Removal of the acetone from the mother liquor yielded another 26 gm. For analysis, the substance was recrystallized from acetone and water. M.p., 180° (with decomposition) (corrected).

$C_{21}H_{22}O_5N_2 \cdot H_2O$ .	Calculated.	C 67.0, H 5.4, N 6.2, $H_2O$ 4.0
448.4	Found.	" 67.1, " 5.4, " 6.2, " 4.4

The substance is rather soluble in acetone, ethanol, and methanol; much less soluble in water and in ethyl acetate.

*Acetyldehydroleucine*—40 gm. of chloroacetyl-*L*-leucine (11) were agitated with 60 cc. of acetic anhydride in a bath at 60° for 1½ hours. On fractiona-

tion *in vacuo*, the azlactone of acetyldehydroleucine distilled at 68-69°, under a pressure of 0.15 mm., as a colorless liquid. The yield was only about 18 gm.

The azlactone was characterized by its transformation into the corresponding acid and its amide. 5 gm. of azlactone were kept overnight at 0° with 12 cc. of water and large colorless crystals formed. Yield, 2.5 gm. They were recrystallized from ethyl acetate. M.p., 155-157° (corrected).

$C_7H_{11}O_2N$ .	Calculated.	C 56.1, H 7.6, N 8.2
171.2	Found.	" 56.0, " 7.7, " 8.2

The amide was obtained by treating at 0° 4.2 gm. of azlactone with 25 cc. of a 10 per cent aqueous solution of ammonia. An oil formed which crystallized on scratching. Yield, 3.5 gm. The amide was recrystallized from 50 per cent ethanol as well formed prisms. M.p., 205-207° (with decomposition) (corrected).

$C_8H_{11}O_2N_2$ .	Calculated.	C 56.4, H 8.3, N 16.4
170.2	Found.	" 56.5, " 8.4, " 16.5

*Acetyldehydroleucylglycine*—8 gm. of acetyldehydroleucine azlactone were treated at 0° with a solution of glycine ethyl ester in ether. Within a few minutes 8.5 gm. of acetyldehydroleucylglycine ethyl ester deposited. It was recrystallized from ethyl acetate. M.p., 130-132° (corrected).

$C_{12}H_{20}O_4N_2$ .	Calculated.	C 56.2, H 7.9, N 10.9
256.3	Found.	" 56.1, " 7.8, " 10.9

The ester dissolves easily in ethyl acetate, ethanol, and methanol; very slightly in ether.

In order to saponify the ester group, 120 cc. of *N* NaOH were added with stirring to a suspension of 30 gm. of ester in 30 cc. of ethanol. After 30 minutes, 120 cc. of *N* HCl were added to the clear solution and the liquid was evaporated to dryness. The colorless residue is a mixture of acetyldehydroleucylglycine and sodium chloride.

For the isolation of pure acetyldehydroleucylglycine, a 5 gm. sample of the aforementioned mixture was dissolved in 50 cc. of water and extracted eight times with 50 cc. portions of ethyl acetate. The combined extracts, on concentration, yielded 2 gm. of acetyldehydroleucylglycine melting at 185-187° (corrected).

$C_{10}H_{16}O_4N_2$ .	Calculated.	C 52.6, H 7.1, N 12.3
228.2	Found.	" 52.6, " 7.2, " 12.2

*Carbobenzoxylglycyl-dl-phenylserine*—The phenylserine ester used in the following experiments was prepared by esterifying *trans*-phenylserine with

ethanol and HCl gas, evaporating *in vacuo*, and repeating the esterification and evaporation. The ester hydrochloride thus obtained melted at 137–139° (corrected). 50 gm. of ester hydrochloride were added with vigorous stirring to an ice-cold mixture of 250 cc. of water containing 40 gm. of  $K_2CO_3$ , and 750 cc. of ethyl acetate. After a few minutes, the ethyl acetate layer was separated and dried over anhydrous  $Na_2SO_4$ . This solution contained 39 to 40 gm. of phenylserine ethyl ester, corresponding to 93 per cent of the theory, and was used directly for the following experiment.

22 gm. of carbobenzoxyglycine chloride (12) were added, with cooling, to a solution of 39 gm. of phenylserine ethyl ester in 750 cc. of ethyl acetate. Phenylserine ester hydrochloride separated out, together with carbobenzoxyglycylphenylserine ethyl ester. The solids were filtered off after 24 hours, dried, and dissolved in 600 cc. of hot 95 per cent ethanol. Addition of 800 cc. of water precipitated 32 gm. of carbobenzoxyglycylphenylserine ethyl ester.<sup>6</sup> After recrystallization from ethanol, the ester melted at 149–151° (corrected).

$C_{21}H_{24}O_6N_2$ .	Calculated.	C 63.0, H 6.0, N 7.0
400.4	Found.	" 63.0, " 6.1, " 7.0

To prepare carbobenzoxyglycylphenylserine, 30 gm. of the ethyl ester were shaken for 1 to 2 hours with a mixture of 80 cc. of *N* NaOH and 160 cc. of methanol. The clear solution was acidified by the addition of 85 cc. of *N* HCl and kept at 0° overnight. Yield, 25.1 gm. The acid was recrystallized from methanol by the addition of water. M.p., 161–163° (corrected).

$C_{19}H_{20}O_6N_2$ .	Calculated.	C 61.3, H 5.4, N 7.5
372.4	Found.	" 61.1, " 5.4, " 7.5

*Carbobenzoxyglycyldehydrophenylalanine*—The azlactone of this compound was obtained when 18.6 gm. of carbobenzoxyglycylphenylserine were kept at room temperature with 95 cc. of acetic anhydride and 2 gm. of anhydrous sodium acetate with frequent shaking. After 24 hours, 100 cc. of ice water were added and, after several hours standing at 0°, the silky needles of the azlactone were filtered off and dried over  $P_2O_5$  and KOH *in vacuo*. Yield, 13.6 gm. For analysis, the almost colorless azlactone was twice recrystallized from ethyl acetate. M.p., 141–142° (corrected).

$C_{19}H_{18}O_6N_2$ .	Calculated.	C 67.8, H 4.8, N 8.3
336.3	Found.	" 67.9, " 4.8, " 8.4

<sup>6</sup> The mother liquor, remaining after the filtration of the dipeptide ester, may be concentrated *in vacuo*. Most of the phenylserine ester contained therein as the hydrochloride may then be recovered by the previously described procedure.

5.5 gm. of azlactone, after being heated in a mixture of 100 cc. of acetone and 20 cc. of water at 60° for 4 hours, yielded, on subsequent evaporation *in vacuo*, 5.6 gm. of carbobenzoxyglycyldehydrophenylalanine which melted at 166–168°. This was recrystallized from ethyl acetate with considerable loss. M.p., 168–170° (corrected).

$C_{15}H_{11}O_4N_1$ .	Calculated.	C 61.4, H 5.1, N 7.9
351.4	Found.	" 61.4, " 5.2, " 7.8

Needles, easily soluble in hot acetone, hot ethyl acetate, and hot ethanol, sparingly soluble in ether, and particularly so in water, were obtained.

In order to obtain the corresponding amide, 3.5 gm. of azlactone were added to a mixture of 7 cc. of a concentrated ammonia solution in water and 4 cc. of pyridine. After 15 hours, 2.5 gm. of amide crystallized on the addition of water and scratching. It was recrystallized from ethyl acetate by the addition of petroleum ether. M.p., 164–166° (corrected).

$C_{14}H_{11}O_4N_2$ .	Calculated.	C 61.6, H 5.4, N 11.9
353.4	Found.	" 61.7, " 5.5, " 11.9

The amide is rather soluble in methanol and ethanol.

*Acetyldehydrophenylalanyl-l-alanine*—This substance was obtained from 8.9 gm. of *l*-alanine, 100 cc. of acetone, 100 cc. of *N* NaOH, and 18.8 gm. of acetyldehydrophenylalanine azlactone. On addition of 102 cc. of *N* HCl, the peptide crystallized as colorless needles. Yield, 17.8 gm. Another considerable quantity was obtained from the mother liquor on evaporation of the acetone *in vacuo*. The peptide was recrystallized from 60 per cent methanol by the addition of more water. M.p., 195–196° (with decomposition) (corrected).

$C_{14}H_{16}O_4N_2$ .	Calculated.	C 60.8, H 5.8, N 10.1
276.3	Found.	" 60.8, " 6.0, " 10.0

$[\alpha]_D^{32} = +69.4^\circ$  (2 per cent, in pyridine). After one more recrystallization  $[\alpha]_D^{33.5}$  was  $+69.6^\circ$ .

*Acetyldehydrophenylalanyl-l-phenylalanine*—This peptide was obtained from a mixture of 8.25 gm. of *l*-phenylalanine, 100 cc. of acetone, 50 cc. of *N* NaOH, and 9.35 gm. of acetyldehydrophenylalanine azlactone by the subsequent addition of 51 cc. of *N* HCl. It was recrystallized three times from 120 cc. of methanol by the addition of 300 cc. of water. Yield, 12.9 gm. M.p., 213–215° (with decomposition) (corrected).

$C_{20}H_{20}O_4N_2$ .	Calculated.	C 68.2, H 5.7, N 8.0
352.4	Found.	" 68.1, " 5.7, " 8.0

$[\alpha]_D^{26} = +35.5^\circ$  (5 per cent, in pyridine). Other preparations gave  $[\alpha]_D^{28} = +35.0^\circ$  and  $+37.6^\circ$ .

*Acetyldehydrophenylalanyl-l-tyrosine*—This substance was described by Bergmann *et al.* (3) as having a melting point of 217–218° (corrected) and  $[\alpha]_D^{20} = +47.1^\circ$  (2.5 per cent in pyridine). The preparation has now been repeated. The melting point of an analyzed sample was found to be 228.5–229.5° (with decomposition) (corrected) after discoloration from 221° on.  $[\alpha]_D^{30} = +45.0^\circ$  (in pyridine).

*Acetyl-dl-phenylalanyldehydrophenylalanine*—20 gm. of acetyl-dl-phenylalanylglycine (9) were shaken for 2 hours with 20 cc. of acetic anhydride, 8.2 cc. of benzaldehyde, and 3.5 gm. of anhydrous sodium acetate. After 2 days the excess anhydride was decomposed by the addition of 20 cc. of ice water. The undissolved, almost colorless crystals (4.5 to 5.0 gm.) were the azlactone of acetyl-dl-phenylalanyldehydrophenylalanine. They were recrystallized first from ethyl acetate, by the addition of petroleum ether, and then from a mixture of 300 cc. of acetone and 10 cc. of water, by the subsequent addition of 1 liter of water. Yield, 3.2 gm. M.p., 206–207° (corrected).

$C_{20}H_{18}O_4N_2$ .	Calculated.	C 71.8, H 5.4, N 8.4
334.4	Found.	" 71.6, " 5.4, " 8.5

2.1 gm. of azlactone dissolved when shaken with a mixture of 20 cc. of acetone and 7 cc. of N NaOH. After 6 hours the solution was filtered and 7.2 cc. of N HCl were added. 2.1 gm. of acetyl-dl-phenylalanyldehydrophenylalanine crystallized as colorless needles. They were dissolved in 14 cc. of hot, 85 per cent methanol and crystallized on cooling and the addition of more water. M.p., 209–211° (with decomposition) (corrected), after sintering at 206°.

$C_{20}H_{20}O_4N_2$ .	Calculated.	C 68.2, H 5.7, N 8.0
352.4	Found.	" 68.1, " 5.8, " 7.8

*Acetyldehydrophenylalanyldehydrophenylalanine*—65 gm. of acetyldehydrophenylalanylglycine were thoroughly mixed with 75 cc. of acetic anhydride, 30 cc. of benzaldehyde, and 17 gm. of anhydrous sodium acetate and kept for 2 days at room temperature. The excess acetic anhydride was then decomposed with 75 cc. of water at 0°. On filtration, 60 gm. of the azlactone of acetyldehydrophenylalanyldehydrophenylalanine were obtained. This was recrystallized from a mixture of acetone and water. M.p., 184–186° (corrected).<sup>7</sup> A mixture with the previously described

<sup>7</sup> The mother liquor, on further dilution with water, yielded a small second crop. When this was covered with the 15-fold amount of ether, bright yellow and deep orange crystals slowly appeared, which were separated mechanically. The yellow crystals were the azlactone described above, m.p. 184–186°. The orange crystals were an isomer, which, above 172°, sintered and melted somewhat to form a yellow semisolid, which in turn melted sharply at 191–193° (with decomposition) (corrected). Found, C 72.0, H 4.7, N 8.3.



azlactone obtained from acetyldehydrophenylalanylphenylserine melted at the same temperature.

$C_{20}H_{16}O_3N_2$ .	Calculated.	C 72.3, H 4.8, N 8.4
332.3	Found.	" 72.1, " 4.9, " 8.5

For hydrolysis to the corresponding acid, 30 gm. of the azlactone were dissolved in a mixture of 300 cc. of acetone and 125 cc. of  $N$  NaOH. After 2 hours, 130 cc. of  $N$  HCl were added. Needles of a bright buff color precipitated. They were dissolved in a boiling mixture of 500 cc. of ethanol and 230 cc. of water and precipitated by the addition of more water at  $0^\circ$ . Yield, 28.0 gm. M.p.,  $204-205^\circ$  (with decomposition) (corrected).

$C_{20}H_{18}O_4 \cdot H_2O$ .	Calculated.	C 65.2, H 5.5, N 7.6, $H_2O$ 4.9
368.4	Found.	" 65.2, " 5.6, " 7.7, " 5.0

Acetyldehydrophenylalanyldehydrophenylalanine forms rods. It is somewhat soluble in hot acetone and hot alcohol, much less so in ethyl acetate, ether, and water.

The amide of this peptide was obtained by shaking 10 gm. of azlactone with a mixture of 20 cc. of pyridine and 80 cc. of concentrated aqueous ammonia. After 3 hours water was added, and the amide filtered off and recrystallized from a 1:2 mixture of alcohol and water. The amide forms coarse four- and six-sided plates. M.p.,  $229^\circ$  (with decomposition) (corrected).

$C_{20}H_{16}N_3O_3$ .	Calculated.	C 68.7, H 5.5, N 12.0
349.4	Found.	" 68.5, " 5.4, " 11.9

*Benzoyldehydrophenylalanyldehydrophenylalanine*—33 gm. of benzoyldehydrophenylalanylglycine were thoroughly mixed at room temperature with 100 cc. of acetic anhydride, 15 cc. of benzaldehyde, and 10 gm. of anhydrous sodium acetate. After 1 day, the resulting azlactone was isolated in the usual manner. It was dried, covered with 100 cc. of ethyl acetate overnight at  $0^\circ$ , filtered, and treated with another 100 cc. of ethyl acetate in a similar manner. Yield, 18 gm. M.p.,  $188-190^\circ$  (corrected). For analysis the azlactone was recrystallized from hot ethyl acetate.

$C_{25}H_{18}O_3N_2$ .	Calculated.	C 76.1, H 4.6, N 7.1
394.4	Found.	" 76.3, " 4.6, " 7.2

The same azlactone was obtained when 20 gm. of benzoyldehydrophenylalanylphenylserine were kept for 24 hours at  $20^\circ$  with 50 cc. of acetic anhydride and 1 gm. of anhydrous sodium acetate and the azlactone then isolated in the usual manner. Yield, 17 gm. After recrystallization from ethyl acetate, 12 gm. melting at  $189-190^\circ$  (corrected) were obtained. Found, C 76.1, H 4.8, N 7.2.

20 gm. of azlactone were shaken at room temperature with 200 cc. of acetone and 55 cc. of  $N$  NaOH until a clear solution resulted. After 5 hours, 57 cc. of  $N$  HCl were added and the acetone removed from the solution *in vacuo*. On addition of water and a small amount of ether, 20.7 gm. of benzoyldehydrophenylalanyldehydrophenylalanine were obtained. The crude material was twice recrystallized by dissolving it in about 500 cc. of hot ethyl acetate and precipitating with petroleum ether. Yield, 12.0 gm. M.p.,  $180-181^{\circ}$  (with decomposition) (corrected).

$C_{25}H_{20}O_4N_2$ .	Calculated.	C 72.8, H 4.9, N 6.8
412.4	Found.	" 72.7, " 5.0, " 6.6

Benzoyldehydrophenylalanyldehydrophenylalanine forms cream-colored rods, which are sparingly soluble in acetone, ethyl acetate, ethanol, and ether, and much less so in petroleum ether and in water.

The amide of benzoyldehydrophenylalanyldehydrophenylalanine was obtained when 6 gm. of the azlactone were added to a mixture of 15 cc. of concentrated aqueous ammonia and 12 cc. of pyridine. After several hours, the amide was precipitated with water, and recrystallized from methanol by the addition of water. M.p.,  $199^{\circ}$  (corrected).

$C_{25}H_{21}O_5N_3$ .	Calculated.	C 73.0, H 5.1, N 10.2
411.4	Found.	" 72.9, " 5.2, " 10.1

Acetyldehydrophenylalanyldehydrotyrosine—26 gm. of acetyldehydrophenylalanylglycine were mixed with 50 cc. of acetic anhydride, 14 gm. of *p*-hydroxybenzaldehyde, and 7 gm. of anhydrous sodium acetate. After initial cooling, the mixture was kept at  $40^{\circ}$  for 2 days. The crude azlactone, isolated in the usual manner, was digested with 150 cc. of acetone at  $0^{\circ}$ , filtered off, and recrystallized from ethyl acetate by the addition of petroleum ether. Yield, 20 gm. The azlactone sintered at  $165^{\circ}$  and melted at  $193-194^{\circ}$  (corrected). As was to be expected, the azlactone contained the phenolic hydroxyl of the tyrosine residue in the acetylated form.

$C_{22}H_{18}O_5N_2$ .	Calculated.	C 67.7, H 4.6, N 7.2
390.4	Found.	" 67.6, " 4.8, " 7.2

39 gm. of azlactone were stirred at room temperature with 250 cc. of acetone and 300 cc. of  $N$  NaOH. After 3 to 4 hours, 305 cc. of  $N$  HCl were added and the acetone removed *in vacuo*. An oil separated which solidified on addition of a small amount of ether. The solid was filtered off by suction. Yield, 28.4 gm. The dark brown crystals were digested for 24 hours at  $0^{\circ}$  with 125 cc. of anhydrous acetone, filtered, and again treated with acetone. The resulting buff crystals, 25 gm., were recrystallized from 1.5 liters of absolute ethanol with the addition of much petro-

leum ether. Needles, melting at  $218^{\circ}$  (with decomposition) (corrected) were obtained.

$C_{20}H_{18}O_5N_2 \cdot \frac{1}{2}H_2O$ .	Calculated.	C 64.8, H 4.9, N 7.6, $H_2O$ 1.2
370.9	Found.	" 64.9, " 5.1, " 7.6, " 1.2

*Benzoyldehydrophenylalanyldehydrotyrosine*—The acetylated azlactone of this compound was prepared from 50 gm. of benzoyldehydrophenylalanylglycine, 150 cc. of acetic anhydride, 23 gm. of *p*-hydroxybenzaldehyde, and 18 gm. of anhydrous sodium acetate. The crude acetyl azlactone was twice digested with 250 cc. of anhydrous acetone at  $0^{\circ}$ . Yield, 55 gm. It was dissolved in 4 liters of hot acetone and crystallized by addition of 5 liters of water. For analysis, a sample was twice recrystallized from much ethyl acetate. M.p.,  $231-233^{\circ}$  (corrected).

$C_{27}H_{20}O_5N_2$ .	Calculated.	C 71.7, H 4.4, N 6.2
452.4	Found.	" 71.6, " 4.4, " 6.3

To a suspension of 5.5 gm. of acetyl azlactone in acetone, 2 equivalents of *N* NaOH were added. The clear solution was acidified after 2 hours and concentrated *in vacuo*. 2.5 gm. of a yellow precipitate were obtained, representing the acetyl-free azlactone. Twice recrystallized from a mixture of acetone and water, this azlactone melted at  $235-238^{\circ}$  (corrected).

$C_{25}H_{18}O_4N_2$ .	Calculated.	C 73.2, H 4.4, N 6.8
410.4	Found.	" 72.8, " 4.6, " 6.9

In order to prepare benzoyldehydrophenylalanyldehydrotyrosine, 9 gm. of acetyl azlactone, suspended in 160 cc. of acetone, were hydrolyzed by 80 cc. of *N* NaOH (4 equivalents). After 3 hours, 82 cc. of *N* HCl and 160 cc. of water were added. The filtered precipitate was dissolved in 85 cc. of acetone and reprecipitated by the addition of 170 cc. of water. Yield, 7.2 gm. M.p.,  $164-166^{\circ}$  (with decomposition) (corrected).

$C_{28}H_{20}O_5N_2 \cdot H_2O$ .	Calculated.	C 67.2, H 5.0, N 6.3, $H_2O$ 4.0
446.4	Found.	" 67.2, " 4.9, " 6.3, " 4.0

The acid forms almost colorless crystals, showing a slight brownish cast. It is soluble in aqueous  $KHCO_3$  and also in methanol, ethanol, and acetone.

The amide of this acid was obtained when 12 gm. of acetyl azlactone were mixed with 25 cc. of ether and 25 cc. of a concentrated aqueous solution of ammonia and allowed to stand overnight. After addition of water, the amide was filtered off (8.3 gm.) and first recrystallized from acetic acid and water and afterwards from methanol and water. M.p.,  $228^{\circ}$  (with decomposition) (corrected).

$C_{25}H_{21}O_4N_3 \cdot H_2O$ .	Calculated.	C 67.4, H 5.2, N 9.4
445.4	Found.	" 67.4, " 5.2, " 9.6

*Acetyldehydrocucyldehydrophenylalanine*—15 gm. of the aforementioned mixture of acetyldehydrocucylglycine and sodium chloride were thoroughly mixed with 25 cc. of acetic anhydride, 7 cc. of benzaldehyde, and 4 gm. of anhydrous sodium acetate. The mixture was initially cooled to counteract the spontaneous generation of heat, then kept at 40°. After about 20 hours, 30 cc. of ice water were added and the azlactone of acetyldehydrocucyldehydrophenylalanine soon crystallized. It was recrystallized from acetone by the addition of water as colorless, rectangular plates, which, in aggregate, showed a slight brownish tint. Yield, 10.0 gm. M.p., 171–173° (corrected).

$C_{17}H_{14}O_2N_2$	Calculated.	C 68.4, H 6.1, N 9.4
293.3	Found.	" 68.5, " 6.2, " 9.2

The azlactone was soluble in ethyl acetate, acetone, and ethanol; very slightly so in water.

The transformation into the free acid was effected by dissolving 10 gm. of azlactone in 35 cc. of acetone and adding 35 cc. of *N* NaOH. After 2 hours, the addition of 35 cc. of *N* HCl produced a yellowish precipitate. It was recrystallized from acetone by the addition of water. Yield, 8.1 gm. of fine, long needles. M.p., 215–216° (with decomposition) (corrected).

$C_{17}H_{14}O_4N_2$	Calculated.	C 64.5, H 6.4, N 8.8
316.3	Found.	" 64.7, " 6.5, " 9.0

*Carbobenzoxylglycyldehydrophenylalanyl-l-glutamic Acid*—3.0 gm. of glutamic acid were shaken with 18 cc. of acetone, 18 cc. of 2 *N* NaOH, and 6.85 gm. of carbobenzoxylglycyldehydrophenylalanine azlactone until a clear solution resulted. Upon addition of 36 cc. of *N* HCl, the carbobenzoxyltripeptide obtained crystallized as colorless rods. Yield, 95 per cent of the theory. M.p., 158–162° (corrected). The tripeptide was dissolved in 30 cc. of a 4:1 acetone-water mixture and reprecipitated by the addition of more water. M.p., 177–179° (with decomposition) (corrected).

$C_{24}H_{23}O_5N_3$	Calculated.	C 59.6, H 5.2, N 8.7
483.5	Found.	" 59.7, " 5.2, " 8.5

$[\alpha]_D^{28} = -29.6^\circ$  (4.8 per cent, in pyridine). After another recrystallization,  $[\alpha]_D^{30} = -28.0^\circ$ .

The closely related peptides glycyldehydrophenylalanine-*l*-glutamic acid, glycyl-*l*-phenylalanyl-*l*-glutamic acid, and glycyl-*d*-phenylalanyl-*l*-glutamic acid have been obtained from chloroacetylphenylserine as starting material (1).

*Carbobenzoxylglycyldehydrophenylalanylphenylserine*—22 gm. of carbobenzoxylglycyldehydrophenylalanine azlactone were added to a solution of 12 gm. of phenylserine in 70 cc. of acetone and 68 cc. of *N* NaOH. The

mixture was shaken until a clear solution resulted and then allowed to stand overnight. On addition of 800 cc. of water and 70 cc. of  $N$  HCl, an oil formed which crystallized at  $0^{\circ}$ . The dried material was dissolved in ethyl acetate and precipitated by the addition of ligroin. 26.4 gm. of carbobenzoxyglycyldehydrophenylalanylphenylserine were thus obtained. For analysis, the substance was recrystallized from ethanol. M.p.,  $168-170^{\circ}$  (corrected).

$C_{28}H_{27}O_7N_3$ .	Calculated.	C 65.0, H 5.2, N 8.1
517.5	Found.	" 64.8, " 5.2, " 8.0

*Acetylbis(dehydrophenylalanyl)glycine*—This acetylated tripeptide was prepared by stirring 4.7 gm. of glycine with 100 cc. of acetone, 62.5 cc. of  $N$  NaOH, and 20.7 gm. of acetyldehydrophenylalanyldehydrophenylalanine azlactone. After 3 to 4 hours the clear solution was acidified with 65 cc. of  $N$  HCl. At  $0^{\circ}$ , 24.5 gm. of the tripeptide crystallized as heavy plates. It was dissolved in a mixture of 600 cc. of water and 250 cc. of methanol by the addition of 12 gm. of sodium bicarbonate. On addition of HCl and some ether, the peptide crystallized. It was recrystallized once more by dissolving it in a mixture of 1 liter of hot methanol and 100 cc. of water. On addition of 1 liter of water, 21.7 gm. of peptide were obtained. For analysis, it was dried at  $100^{\circ}$  and 1 mm. over  $P_2O_5$ .

$C_{22}H_{21}O_5N_3$ .	Calculated.	C 64.8, H 5.2, N 10.3
407.4	Found.	" 64.7, " 5.2, " 10.1

The acetylated tripeptide begins to discolor at  $205^{\circ}$  and decomposes completely at  $216^{\circ}$  (corrected). It is rather sparingly soluble in most solvents.

*Acetylbis(dehydrophenylalanyl)-l-alanine*—5.6 gm. of *l*-alanine were stirred for 1 hour with 100 cc. of acetone, 63 cc. of  $N$  NaOH, and 20.7 cc. of acetyldehydrophenylalanyldehydrophenylalanine azlactone. On the addition of 65 cc. of  $N$  HCl, 23.3 gm. of colorless needles were obtained. They were dissolved in 100 cc. of hot methanol. On the addition of 200 cc. of water and some ether, there crystallized at  $0^{\circ}$  20.5 gm. of the acetyl tripeptide. For analysis it was recrystallized once more. M.p.,  $215-216^{\circ}$  (with decomposition) (corrected).

$C_{23}H_{23}O_5N_3$ .	Calculated.	C 65.5, H 5.5, N 10.0
421.4	Found.	" 65.8, " 5.7, " 9.9

$[\alpha]_D^{20} = -268.4^{\circ}$  (2.3 per cent, in pyridine). A sample which was recrystallized once more showed  $[\alpha]_D^{30} = -255.1^{\circ}$ . This preparation showed at a lower temperature  $[\alpha]_D^{20} = -282.9^{\circ}$ .

*Acetylbis(dehydrophenylalanyl)-l-leucine*—This peptide was obtained from 4.2 gm. of *l*-leucine, 85 cc. of acetone, 35 cc. of  $N$  NaOH, and 11 gm.

of acetyldehydrophenylalanyldehydrophenylalanine azlactone. On addition of 36 cc. of  $N$  HCl to the resulting solution, 15.1 gm. of peptide were precipitated as creamy white rods. It was twice recrystallized by dissolving it in 1.2 liters of boiling 95 per cent ethanol and adding to this solution 3 liters of water. M.p., 235–236° (with decomposition) (corrected), after sintering from 225°.

$C_{24}H_{29}O_5N_3$ .	Calculated.	C 67.4, H 6.3, N 9.1
463.5	Found.	" 67.4, " 6.3, " 9.2

$[\alpha]_D^{32} = -245.2^\circ$  (2 per cent, in pyridine). After another recrystallization  $[\alpha]_D^{32} = -245.6^\circ$ .

For acetyldehydrophenylalanylleucine, no rotation was given by Behrens *et al.* (4). For an analyzed preparation, there has now been found  $[\alpha]_D^{31} = +36.9^\circ$  (2.5 per cent, in pyridine). A sample which was recrystallized once more showed  $[\alpha]_D^{31} = +37.2^\circ$ .

*Acetylbis(dehydrophenylalanyl)-l-phenylalanine*—5.3 gm. of *l*-phenylalanine were stirred with 85 cc. of acetone, 34 cc. of  $N$  NaOH, and 11 gm. of acetyldehydrophenylalanyldehydrophenylalanine azlactone. At 30°, the azlactone dissolved in  $\frac{1}{2}$  to 1 hour. After another hour, the solution was cooled and acidified with 35 cc. of  $N$  HCl. The acetyl tripeptide crystallized at once. Yield, 15.6 gm. It was dissolved in 1.7 liters of hot 90 per cent ethanol and reprecipitated by the addition of water. Yield, 14.0 gm. M.p., 229–230° (with decomposition) (corrected), after darkening from 216° on.

$C_{23}H_{27}O_5N_3$ .	Calculated.	C 70.0, H 5.5, N 8.4
497.5	Found.	" 69.9, " 5.5, " 8.4

$[\alpha]_D^{28} = -172.2^\circ$  (5 per cent, in pyridine). Another preparation showed  $[\alpha]_D^{28} = -171.6^\circ$ .

*Acetylbis(dehydrophenylalanyl)-l-tyrosine*—To a suspension of 11.6 gm. of *l*-tyrosine in 170 cc. of acetone, were added, with stirring, 68 cc. of  $N$  NaOH and then, 22 gm. of acetyldehydrophenylalanyldehydrophenylalanine azlactone. After 5 hours stirring at 30°, the mixture was filtered, the filter repeatedly washed with water, and the combined filtrates acidified by the addition of 70 cc. of  $N$  HCl. At 0°, 26.4 gm. of crystals separated. They were dissolved in 100 cc. of hot methanol, the solution was filtered, and 400 cc. of water and 200 cc. of ether were added to the filtrate. The peptide crystallized at 0°. M.p., 172–173.5° (with decomposition) (corrected).

$C_{29}H_{37}O_6N_3 \cdot \frac{1}{2}H_2O$ .	Calculated.	C 66.6, H 5.4, N 8.0, $H_2O$ 1.7
522.5	Found.	" 66.4, " 5.6, " 7.9, " 1.7

$[\alpha]_D^{30} = -137.1^\circ$  (2.1 per cent, in pyridine). The preparation was recrystallized once more.  $[\alpha]_D^{33} = -133.6^\circ$ .

*Acetylbis(dehydrophenylalanyl)-l-proline*—12 gm. of *l*-proline were dissolved in a mixture of 100 cc. of acetone and 108 cc. of *N* NaOH and, after addition of 34.7 gm. of acetyldehydrophenylalanyldihydrophenylalanine azlactone, stirred for an hour. On addition of 110 cc. of *N* HCl, an oil separated which soon crystallized. Yield, 43 gm. For recrystallization, the product was dissolved in 200 cc. of methanol and 200 cc. of water were added. At 0°, 38 gm. of needle-shaped crystals were obtained. M.p., 203–204° (with decomposition) (corrected). For analysis, the substance was dried at 78° and 0.05 mm. over anhydrous CaSO<sub>4</sub>.

C <sub>25</sub> H <sub>25</sub> O <sub>5</sub> N <sub>3</sub> .	Calculated.	C 67.1, H 5.6, N 9.4
447.4	Found.	" 66.7, " 5.6, " 9.5

$[\alpha]_D^{10} = +60.6^\circ$  and  $[\alpha]_D^{20} = +50.6^\circ$  (2 per cent, in pyridine). Another preparation gave  $[\alpha]_D^{30} = +48.8^\circ$ .

In a previous communication (Behrens *et al.* (4)), acetyldehydrophenylalanyl-*l*-proline was described. The rotation of this substance has now been determined. For two preparations the values  $[\alpha]_D^{32} = +79.9^\circ$  and  $[\alpha]_D^{32} = +80.5^\circ$ , respectively, were found.

*Acetylbis(dehydrophenylalanyl)phenylserine*—18 gm. of *trans*-phenylserine were stirred at 50° with 150 cc. of acetone, 100 cc. of *N* NaOH, and 33.2 gm. of acetyldehydrophenylalanyldihydrophenylalanine azlactone. After 1 to 2 hours, the clear solution was cooled and acidified with 105 cc. of *N* HCl. On standing at 0°, the acetylated tripeptide crystallized. Yield, 44.5 gm. For analysis, a sample was recrystallized several times from hot 90 per cent ethanol by the subsequent addition of water and dried at 120° and 1 mm. over anhydrous CaSO<sub>4</sub>. M.p., 223–225° (with decomposition) (corrected).

C <sub>29</sub> H <sub>27</sub> O <sub>5</sub> N <sub>3</sub> .	Calculated.	C 67.8, H 5.3, N 8.2
513.5	Found.	" 67.9, " 5.4, " 8.2

*Acetylbis(dehydrophenylalanyl)-l-glutamic Acid*—This substance was prepared by employing 30 gm. of *l*-glutamic acid, 450 cc. of acetone, 410 cc. of *N* NaOH, and 66 gm. of the azlactone of acetyldehydrophenylalanyldihydrophenylalanine. After 2 hours stirring, 415 cc. of *N* HCl, 100 cc. of ether, and 350 cc. of water were added to the clear solution. Yield, 90 gm. For purification, the substance was dissolved in methanol and precipitated by the addition of water and ether. The long, colorless needles melted at 209–210° (with decomposition) (corrected).

C <sub>25</sub> H <sub>25</sub> O <sub>7</sub> N <sub>3</sub> .	Calculated.	C 62.6, H 5.2, N 8.8
479.5	Found.	" 62.8, " 5.5, " 8.8

$[\alpha]_D^{20} = -182.4^\circ$ . After recrystallization,  $[\alpha]_D^{30} = -182.6^\circ$ .

*Bis(acetyldehydrophenylalanyl)-l-cystine*—A suspension of 6 gm. of *l*-

cystine in a mixture of 20 cc. of acetone and 25 cc. of water was stirred with 52 cc. of  $N$  NaOH and 9.5 gm. of acetyldehydrophenylalanine azlactone for about 5 hours. After filtration and the addition of 30 cc. of water and 55 cc. of  $N$  HCl, the peptide crystallized as colorless microscopic needles. Yield, 14.3 gm. For recrystallization, the peptide was dissolved in 80 cc. of hot 65 per cent methanol and precipitated by the addition of water. M.p., 212–213° (with decomposition) (corrected).

$C_{23}H_{30}O_8N_4S_2$ .	Calculated.	C 54.7, H 4.9, N 9.1, S 10.4
614.7	Found.	" 54.5, " 4.8, " 9.1, " 10.4

$[\alpha]_D^{30.5} = -19.5^\circ$  (2 per cent, in pyridine). After another recrystallization  $[\alpha]_D^{34}$  was  $-19.0^\circ$ .

*Azlactone of Acetylbis(dehydrophenylalanyl)dehydrophenylalanine*—20 gm. of acetylbis(dehydrophenylalanyl)phenylserine were treated with 50 cc. of acetic anhydride and 2 gm. of sodium acetate for 2 days at room temperature. 50 cc. of ice water were added. After the mixture had been kept at 0° for 24 hours, the yellow azlactone of acetylbis(dehydrophenylalanyl)dehydrophenylalanine was filtered. Yield, 18 gm. The azlactone was recrystallized several times by dissolving it in 1.25 liters of boiling acetone and precipitating it by the addition of 900 cc. of water. Yield, 11.5 gm. M.p., 233–235° (with decomposition) (corrected).

$C_{23}H_{23}O_8N_4$ .	Calculated.	C 72.9, H 4.8, N 8.8
477.5	Found.	" 72.9, " 4.8, " 8.9

*Acetyltris(dehydrophenylalanyl)-l-phenylalanine*—10.6 gm. of *l*-phenylalanine from hemoglobin (13) were suspended in 200 cc. of acetone. 70 cc. of  $N$  NaOH were added with stirring and subsequently 31.6 gm. of acetylbis(dehydrophenylalanyl)dehydrophenylalanine azlactone. After 1 to 2 hours stirring at 40°, the solution was cooled, and 73 cc. of  $N$  HCl and 50 cc. of ether were added. Crystallization soon started. Yield, at 0°, 29.4 gm. The yellow substance was heated with 250 cc. of absolute alcohol, an undissolved yellow substance filtered off, and the filtrate diluted with 1 liter of water and some ether. The tetrapeptide crystallized in four-sided platelets. After a second recrystallization, it was colorless and weighed 19.5 gm. The peptide turns yellow at 172–173° and melts to a viscous half solid mass which decomposes completely at 201–202° (corrected).

$C_{38}H_{44}O_8N_4$ .	Calculated.	C 71.0, H 5.3, N 8.7
642.7	Found.	" 71.1, " 5.4, " 8.6

$[\alpha]_D^{30} = -36.3^\circ$  (2.3 per cent, in pyridine). After another recrystallization  $[\alpha]_D^{30}$  was  $-35.4^\circ$ .

*Acetyltris(dehydrophenylalanyl)phenylserine*—To a solution of 20 gm.



of *trans*-phenylserine in 450 cc. of acetone, 50 cc. of water, and 110 cc. of *N* NaOH, 48 gm. of the azlactone of bis(dehydrophenylalanyl)dehydrophenylalanine were added, and the mixture was stirred at 50°. After 9 hours the material, which contained large amounts of orange needles, was cooled and acidified by the addition of 110 cc. of *N* HCl. Yield, 60.5 gm. The substance was dissolved in 250 to 300 cc. of methanol, some undissolved part filtered off, and the filtrate mixed with 750 cc. of water and 250 cc. of ether. The crystals obtained were recrystallized a second time in the same manner (56 gm.), and subsequently twice dissolved in a hot 1:1 mixture of ethanol and ethyl acetate, and crystallized by the addition of petroleum ether. Yield, 36 gm. of six-sided platelets, melting at 199° (with decomposition) (corrected). The air-dry substance was analyzed.

$C_{38}H_{34}O_7N_4 \cdot H_2O$	Calculated.	C 67.4, H 5.4, N 8.3
676.7	Found.	" 67.2, " 5.3, " 8.2

An attempt to determine the water of crystallization and to analyze the anhydrous substance gave somewhat ambiguous results. The exact composition of the air-dry substance can therefore not be regarded as definitely ascertained.

*Azlactone of Acetyltris(dehydrophenylalanyl)dehydrophenylalanine*—The azlactone was obtained by shaking 25 gm. of the corresponding tetrapeptide of phenylserine described above with 75 cc. of acetic anhydride and 2 gm. of anhydrous sodium acetate. After 24 hours, 75 cc. of ice water were added. The mixture was kept for several hours at 0°. The filtered azlactone was then recrystallized from 850 cc. of acetone by the addition of water. Yield, 21 gm. After a second recrystallization, the azlactone melted at 247–249° (with decomposition) (corrected).

$C_{38}H_{30}O_6N_4$	Calculated.	C 73.3, H 4.8, N 9.0
622.6	Found.	" 73.2, " 5.1, " 8.7

*Bis(acetyldehydrophenylalanyldehydrophenylalanyl)-L-cystine*—12 gm. of *L*-cystine were stirred with 200 cc. of acetone and 110 cc. of *N* NaOH. After a short while, 33 gm. of acetyldehydrophenylalanyldehydrophenylalanine azlactone were added. After 2 to 3 hours, the solution was filtered and 120 cc. of *N* HCl were added followed by some ether. Soon colorless crystals separated. Yield, 43.4 gm. For recrystallization they were dissolved in a mixture of 500 cc. of water and 150 cc. of alcohol with the addition of 25 gm. of  $NaHCO_3$ . The filtered solution was, after the addition of ether, acidified with HCl. Yield, 41.3 gm. M.p., 209–211° (corrected).

$C_{46}H_{44}O_{10}N_6S_2 \cdot 2H_2O$	Calculated.	C 58.8, H 5.1, N 8.9, S 6.8
941.1	Found.	" 58.9, " 5.2, " 8.9, " 6.8

$[\alpha]_D^{30} = -82.3^\circ$  and  $[\alpha]_D^{20} = -86.1^\circ$  (2 per cent, in pyridine). These values were obtained with the same solution. In another sample,  $[\alpha]_D^{32}$  was  $-82.6^\circ$ .

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# THE UTILIZATION OF $\beta$ -HYDROXYBUTYRIC ACID BY THE PERFUSED LACTATING MAMMARY GLAND

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It was reported previously on the basis of arteriovenous differences that the lactating mammary gland of the normal cow utilizes  $\beta$ -hydroxybutyric acid (Shaw and Knodt (1)). Later, Shaw (2) reported that the utilization of this substance by the mammary gland increased more than 100 per cent in ketosis when the blood  $\beta$ -hydroxybutyric acid was abnormally high. As much as 37 per cent of the oxygen consumption of the gland of the normal cow could be accounted for by the oxidation of  $\beta$ -hydroxybutyric acid. In ketosis the utilization of this substance was so great that practically all of the oxygen consumed by the mammary gland could be accounted for when it was postulated that the  $\beta$ -hydroxybutyric acid was oxidized by the mammary tissue for energy purposes.

The object of the experiments reported in this communication was to ascertain whether the foregoing findings could be confirmed by a different method. In addition it was desired to determine whether the large utilization of  $\beta$ -hydroxybutyric acid observed in ketosis would occur when there was a normal quantity of glucose in the blood.

## EXPERIMENTAL

The experiments were carried out on bovine mammary glands perfused with blood according to the technique described by Petersen, Shaw, and Visscher (3). Chlorazol fast pink was used as an anticoagulant. 2 to 10 gm. of sodium  $\beta$ -hydroxybutyrate<sup>1</sup> were added to the blood at the beginning of each experiment. Acetone bodies were determined by the method of Barnes and Wick (4), and blood oxygen was determined by the method of Van Slyke and Neill (5).

Nine perfusion experiments were conducted in which sodium  $\beta$ -hydroxybutyrate was added to the perfusate at the beginning of the perfusion. In each experiment the blood was analyzed for acetone bodies at the beginning, after the addition of the  $\beta$ -hydroxybutyrate, and at the end of the experi-

<sup>1</sup> The racemic salt was kindly furnished us by Dr. R. H. Barnes of the University of Minnesota.

ment to determine the total uptake of acetone bodies by the gland. The blood glucose was maintained at or in excess of 40 mg. per cent by the addition of a 50 per cent solution of glucose from time to time during the perfusion, the amount added being estimated by the rate of blood flow through the gland.

The results are summarized in Table I. In the nine experiments there was an average uptake of 2089.4 mg. of  $\beta$ -hydroxybutyric acid by the glands. The fraction consisting of acetone and acetoacetic acid was apparently not utilized. As considerable sodium  $\beta$ -hydroxybutyrate was added to the perfusate at the beginning of each perfusion, it was to be expected that some of the  $\beta$ -hydroxybutyrate would diffuse into the mam-

TABLE I  
*Utilization of Added  $\beta$ -Hydroxybutyrate by Perfused Lactating Mammary Gland*

Perfusion No.	$\beta$ -Hydroxybutyric acid			Acetone + acetoacetic acid (as acetone)			Times blood traversed gland
	Beginning perfusate	Final perfusate	Total disappearance	Beginning perfusate	Final perfusate	Difference	
	mg. per cent	mg. per cent	mg.	mg. per cent	mg. per cent	mg. per cent	
106	17.77	4.55	1057.6	1.10	1.17	-0.07	4
110	21.98	2.07	1592.8	0.60	0.78	-0.18	13
111	27.35	2.48	1989.6	2.06	1.80	0.26	5
112	32.46	2.75	2079.7	2.23	1.91	0.32	6
114	25.39	0.72	1983.6	1.47	1.62	-0.15	11
115	25.95	1.32	1970.4	1.36	1.78	-0.42	17
126	29.44	1.42	2521.8				16
131	16.65	4.75	1305.4				4
154	81.80	28.00	4304.0				10
Average.....	30.98	5.34	2089.4	1.47	1.51	-0.04	

mary tissue. To determine whether the  $\beta$ -hydroxybutyrate was actually being utilized by the glandular tissues, the entire mammary gland used in Perfusion 131, Table I, was macerated immediately following the perfusion and a weighed aliquot analyzed for acetone bodies. The macerated tissue was immersed in a dilute acid solution, after weighing, to prevent further breakdown of the  $\beta$ -hydroxybutyric acid by enzymatic action. The total acetone body content of the gland at the end of the perfusion, calculated as  $\beta$ -hydroxybutyric acid, was 197.7 mg. The minimum utilization of  $\beta$ -hydroxybutyric acid by the perfused gland was therefore 1107.7 mg.

A more complete balance study was then made in which the total oxygen consumption of the gland was also determined. The left half of the udder was separated from the right half just before the beginning of the perfusion and immediately weighed and macerated and an aliquot immersed in a dilute acid solution. At the end of the perfusion the perfused half was

treated in the same fashion. The total acetone bodies per gm. of tissue (expressed as  $\beta$ -hydroxybutyric acid) were then determined on the two aliquots. Assuming that the concentration of  $\beta$ -hydroxybutyric acid per gm. of tissue in the right gland at the beginning of the perfusion was the same as that of the left gland which had been analyzed, it was possible to determine the quantity of  $\beta$ -hydroxybutyric acid which had been metabolized by the gland during the perfusion. Sufficient  $\beta$ -hydroxybutyrate was added at the beginning to maintain a relatively high concentration of this substance in the blood during the entire perfusion, the concentration being

TABLE II

*Per Cent of Oxygen Uptake Accounted for by  $\beta$ -Hydroxybutyrate Burning by Mammary Gland Perfused with Blood Containing Added  $\beta$ -Hydroxybutyrate (Perfusion 154)*

Time lapse	Volume of blood through gland	Oxygen utilization	
			Total
min.	cc.	vol. per cent	cc.
15	6,315	3.56	222.8
20	8,150	6.25	509.4
20	8,575	6.34	543.7
40	17,075	5.69	971.6
40	18,124	6.14	1112.8
40	27,500	4.61	1267.8
175			4628.1

$\beta$ -Hydroxybutyric acid at beginning, blood, mg. ....	6544.0
“ “ “ “ tissue, “ .....	132.8
“ “ “ “ end, blood, mg.....	2240.0
“ “ “ “ tissue, “ .....	205.8
“ “ “ “ total utilization, mg... ..	4231.0
Assuming $\beta$ -hydroxybutyrate burning, total oxygen required, cc.....	4104.0
“ “ “ “ oxygen consumed needed, % .....	88.7

81.8 mg. per cent at the beginning and 28.0 mg. per cent at the end of the experiment.

The blood flow was measured at 5 minute intervals. The oxygen utilization was determined at the intervals noted in Table II. The blood flow was remarkably constant during the greater part of the perfusion. The oxygen uptake was also quite uniform, except at the very beginning and near the end of the perfusion. The blood was not completely oxygenated at the beginning, and as a result the oxygen uptake was relatively small during the first few minutes. Toward the end of the perfusion the blood flow increased, and the oxygen uptake per unit of volume of blood decreased.

An important question arises as to the percentage of the total oxygen

consumption of the gland that can be accounted for by  $\beta$ -hydroxybutyric acid loss. From the balance studies shown in Table I, it will be seen that the utilization of  $\beta$ -hydroxybutyric acid is quite large when there is a considerable concentration of this substance in the blood. The complete balance study presented in Table II shows that in this experiment 88.7 per cent of the total oxygen consumption can be accounted for by  $\beta$ -hydroxybutyric acid utilization, assuming that it is oxidized for energy purposes and that 1.0 mg. of  $\beta$ -hydroxybutyric acid requires 0.97 cc. of oxygen for complete combustion.

#### DISCUSSION

The studies on the acetone body metabolism of the perfused gland confirm the findings that the lactating mammary gland utilizes  $\beta$ -hydroxybutyric acid. Likewise the data are in agreement with the findings of Shaw (2) that the gland of the cow exhibiting ketonemia used much more  $\beta$ -hydroxybutyric acid than that of the normal cow and that the utilization is sufficient to account for most of the oxygen consumed by the gland.

As the blood glucose is quite low in ketosis in cattle, there was a possibility that the increased utilization of  $\beta$ -hydroxybutyric acid in ketosis was due to the failure of the gland to obtain sufficient glucose for energy purposes and that  $\beta$ -hydroxybutyric acid would be used by the gland in larger quantities only when there was a deficiency of glucose. There was also the alternate possibility that  $\beta$ -hydroxybutyric acid was oxidized in preference to glucose and when present in large quantities would result in a decreased utilization of glucose by the gland. However, Shaw (6) was unable to detect any decrease in the utilization of glucose by the mammary gland of the cow with ketosis. It was suggested (2), therefore, that the mammary gland of the normal cow oxidizes other fat in addition to  $\beta$ -hydroxybutyric acid and that the increase in the utilization of  $\beta$ -hydroxybutyric acid in ketosis was accompanied by a decrease in the oxidation of other fat. Since the gland continues to obtain its normal supply of glucose in ketosis, it appears unlikely that the large increase in the utilization of  $\beta$ -hydroxybutyric acid in ketonemia is due to the oxidation of this substance in preference to glucose. Likewise it appears improbable that the increased utilization is the result of hypoglycemia. The latter observation is substantiated by the data in Tables I and II which show that the gland utilizes  $\beta$ -hydroxybutyric acid in excess of normal even when the blood glucose is maintained at a high level.

It is, of course, possible that  $\beta$ -hydroxybutyric acid is used in direct synthesis. This possibility has been examined by Shaw *et al.* (7), with a negative conclusion.

The bulk of the evidence is believed to favor the suggestion that  $\beta$ -hy-

droxybutyric acid is oxidized by the gland for energy purposes and that the gland of the normal cow also oxidizes other non-carbohydrate substances for energy purposes.

#### SUMMARY

1. Added  $\beta$ -hydroxybutyrate is utilized by the perfused bovine mammary gland.

2. The quantity of  $\beta$ -hydroxybutyric acid metabolized by the gland, perfused with blood containing large quantities of this substance, is considerably in excess of that utilized by the gland of the normal cow and is not dependent upon a low blood sugar level.

3. As much as 88 per cent of the oxygen consumption of the perfused gland may be due to  $\beta$ -hydroxybutyrate burning when the concentration of the latter substance is maintained at or above 28 mg. per cent.

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## A SIMPLIFIED PHOTELOMETRIC ESTIMATION OF TRIGONELLINE\*

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It has been shown by Field, Melnick, Robinson, and Wilkinson (1) that a satisfactory measure of nicotinic acid nutrition will probably require the inclusion of a nearly quantitative method for the estimation of trigonelline, since a major portion of excreted nicotinic acid is generally found as the betaine.

There have been recorded but few reactions offering promise as tools for the estimation of the small concentrations of trigonelline found in urine. The types of treatment which have been investigated for analytical purposes include oxidation (2), alkaline treatment followed by reaction with cyanogen bromide and an amine (3, 4), miscellaneous color reactions (5-7), demethylation and estimation of the released nicotinic acid, and alkaline treatment followed by condensation with an amine (8).

A logical reaction to apply would seem to be the standard alkimide determination (9) followed by the cyanogen bromide estimation of nicotinic acid (3). On pure trigonelline we have been able to secure recoveries of over 90 per cent of trigonelline, as nicotinic acid. Recoveries of trigonelline added to urine, however, were not satisfactory.

The color reaction based on alkaline treatment of trigonelline solutions, which has been described by Kodicek and Wang (8), appeared to be quite adaptable to routine clinical use. Following the directions described, we did not secure consistent results, however, and found it necessary to modify the procedure in several ways. These modifications have resulted in simplification, a saving in time, and relatively precise analyses.

In the procedure of Kodicek and Wang, the material containing trigonelline is heated in alkaline alcohol to yield methylamine as one reaction product. The remaining product condenses with any of various amines to give the dye which is employed as a measure of the trigonelline present. When the ethanolic method was employed in this laboratory, we found that the strong aqueous sodium hydroxide caused separation into two

\* The expenses of this investigation were defrayed by The Upjohn Company, Kalamazoo, and the Horace H. Rackham School of Graduate Studies, University of Michigan, Ann Arbor.

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phases. It seemed likely that this might serve as a primary source of the inconsistent results which we obtained. Other solvents were therefore tried. The color did not develop normally when water was the only solvent. Methanol, used as described in the experimental section, was found to yield a monophasic reaction medium. This alcohol resembled ethanol rather than water in promoting the formation of the chromogenic degradation product. An added advantage of this medium is that the final, neutralized sample seldom exhibits any color, thus eliminating the use of charcoal and its attendant effect upon reproducibility (4).

The need for sulfate removal as practiced when benzidine is employed as the reacting amine was also avoided. Of a number of amines tested under comparable conditions, dianisidine and *p*-aminoacetanilide showed as great or greater sensitivity. Furthermore, they did not precipitate with sulfate.

At the concentrations at which measurements of trigonelline are made in the diluted urines, the photometric intensity-concentration curve approximates a straight line. Such curves were determined for a number of urines and one fairly typical sample was selected for an extended study of this sort. The curves are presented in Fig. 1. It is likely that the value of the photometric intensity relative to the concentration decreases at concentrations higher than those tested. This belief is substantiated by unsuccessful experiments carried out with the purpose of preparing and isolating for characterization the actual pigment formed.

#### EXPERIMENTAL

*Sensitivity of Various Amines*—In these tests 10 mg. of trigonelline acid sulfate (10) were dissolved in 4 cc. of water, and 16 cc. of ethanol were added. To this were added 4.2 cc. of 40 per cent sodium hydroxide solution and the mixture was refluxed for half an hour. The solution was then cooled and neutralized to pH 7 ( $\pm 0.5$ ) with Alkacid paper as indicator, diluted to 1 liter with water, and filtered. To 10.0 cc. portions was added 1.0 cc. of a solution of each of the amines listed below made up to a nearly saturated solution in 5 per cent hydrochloric acid. All amines were Eastman products. Those tested were *p*-aminoacetanilide, *p*-aminoacetophenone, 1-aminobenzimidazole, *p*-aminobenzoic acid, *p*-aminobenzophenone, 1-amino-2-naphthol, 2-amino-4-nitrophenol,  $\alpha$ -aminopyridine, benzidine, cresidine, dianisidine, sulfanilic acid, tolidine, and *p*-toluidine. The intensities of color developed were read on the Evelyn photoelectric colorimeter (11) through Filter 520. Dianisidine proved to be the most sensitive amine.

#### *Reagents*—

Alkali. 400 gm. of sodium hydroxide are dissolved in 600 cc. of water.

Dilute hydrochloric acid. 10 cc. of concentrated HCl are made up to 200 cc. with water.

Methanol. Du Pont synthetic methanol was employed.

Dianisidine solution. 2 gm. of dianisidine, previously twice recrystallized from ethanol, are dissolved in 100 cc. of acetone and 300 cc. of 1.8 per cent hydrochloric acid.

Standard trigonelline solution. 191 mg. of trigonelline sulfate (10) are made up to 2000 cc. with water containing one-fifth volume of methanol. The methanol serves to inhibit bacterial and mold growth.

*Procedure*—In each of two 125 cc. Erlenmeyer flasks are placed 2.50 cc. of the urine. To the test sample 2.50 cc. of water are added, while 1.0 cc. of standard trigonelline solution plus 1.50 cc. of water is added to the recovery sample. 15 cc. of methanol and 5.0 cc. of 40 per cent NaOH solution are added to each. The solutions are refluxed for 30 minutes under condensers with affixed soda lime tubes. The flasks are then cooled and the contents transferred to 50 cc. volumetric flasks. 5 cc. of water are now added to each volumetric flask. The Erlenmeyer flasks are rinsed with methanol and the rinsings also added. The pH is adjusted to 8 with concentrated HCl, care being taken not to allow the solution to become acid. The volume is made up to 50.0 cc. with methanol. From each sample two 9.0 cc. portions of clear liquid are pipetted into two photometer tubes and 1.0 cc. of dianisidine solution is added to one; to the other, which serves as a blank, 1.0 cc. of 1.8 per cent HCl is added. The contents of each tube are thoroughly mixed. The companion blank is used to set the instrument at 100, and the tubes containing dianisidine are read at 10 minute intervals (Filter 520) until a maximum intensity of color is reached.

The difference between the two  $L^1$  values for the recovery sample and the analyzed sample is divided by 10 (the number of micrograms of trigonelline added to the recovery sample) to give  $K$ . The content of the measured fraction of the original sample of urine is then equal to its  $L$  value divided by the calculated  $K$ . The amount of trigonelline in the original 2.50 cc. sample is then 50/9 times this latter amount.

It is necessary to determine the  $K$  each time since it varies for different urines and from the values in aqueous solution.

The color of a blank without urine but including methanolic alkaline treatment, cooling, neutralization, and addition of dianisidine solution was tested against a tube containing water, used to set the instrument at 100. The readings on two such samples were 100 and 102, indicating absence of reagent color. For each determination, a blank can therefore be

<sup>1</sup>  $L$  is analogous to optical density as measured on a spectrophotometer and corresponds to the quantity  $(2 - \log G)$ , in which  $G$  is the galvanometer reading.

simply prepared from the diluted, treated urine without added dianisidine, as described above. With few exceptions, these blanks behave in the photometer in the same way as an equivalent volume of water. The dianisidine solution gradually develops a color. After several weeks it becomes necessary either to correct for this color or to employ a fresh solution.

*Precision*—As a measure of precision of the method, three groups of four analyses were run. One pooled urine was employed for sampling in each group. The following per cent standard deviations were obtained,  $\pm 4.7$ ,  $\pm 3.9$ ,  $\pm 8.0$ .

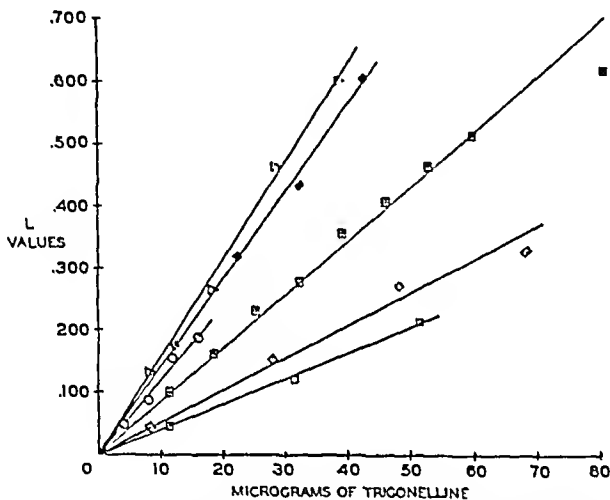


FIG. 1. Degree of deviation from the Bouguer-Beer law of six urine samples. Each line represents the average of the slopes ( $K$ ) of corresponding points.

It is possible for one person to run sixteen samples, including eight recovery samples, in a standard working day.

*Validity of Bouguer-Beer Law*—An experiment was first conducted without urine. The described procedure was followed, except for the amounts of added trigonelline. The solution of trigonelline contained twice as much as the standard, and 0.50, 1.00, 1.50, and 2.00 cc. were added. Instead of urine, each flask contained 2.50 cc. of 1 per cent urea solution for its buffer effect during neutralization. For 10, 20, 30, and 40  $\gamma$  of trigonelline, there were obtained, respectively, for  $K$  0.0227, 0.0206, 0.0218, and 0.0194.

The results on six urinary samples are recorded in Fig. 1.

*Specificity*—Kodicek and Wang found N-methylpyridinium hydroxide to give the same color as trigonelline. Of a number of other compounds tested in this study, only dextrose, in the presence of urea, interfered.

Dextrose is, of course, structurally remote from trigonelline. It is likely, however, that the fundamental color reaction involves formation of a Schiff base, and the ubiquitous dextrose might be expected to give aldehydes in the alkaline treatment. Dextrose solutions were accordingly run through the described procedure. The color values which are presented in Table I indicate a limitation of the analysis in the case of food-stuffs and diabetic urines.

The other compounds tested included nicotinuric acid, nikethamide,  $\alpha$ -picoline, nicotine, caffeine, Neo-iopax, Diodrast, betaine, choline, thymol, urea, pyridoxine betaine, and the methylated nicotine of Pictet and Gnequand (12). The last two compounds were of interest as conceivable excretion products of pyridoxine and nicotine, respectively. The

TABLE I  
*Color Value of Dextrose Solutions of Various Concentrations*

The color is expressed as the number of micrograms of trigonelline per 2.5 cc. to which it is equivalent.

Dextrose solution	Color value
<i>per cent</i>	
1	9
2	13
3	24
4	71

negative behavior of the pyridine compounds in this procedure is in contrast to the activity of such substances with cyanogen bromide (3, 4).

#### SUMMARY

Factors operating in the estimation of trigonelline by alkaline treatment have been isolated and studied, and there has been evolved a simplified procedure for obtaining relatively highly reproducible results. Methanol was found to be a solvent which permits the reaction to be carried out in a single phase. The blank color is thereby diminished, and decolorization by the use of charcoal is eliminated. Dianisidine was found to be an amine condensing to give a dye of high sensitivity. Dianisidine is also usable without previous removal of sulfate ion. The specificity of the fundamental reactions has been studied and dextrose has been found to interfere in determinations by this method.

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# THE SYNTHESIS OF NICOTINURIC ACID\*

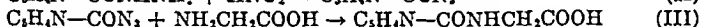
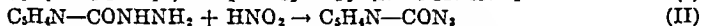
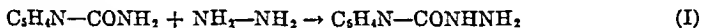
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(Received for publication, September 2, 1942)

In obtaining compounds for the study of nicotinic acid metabolism (1), it was apparent that a convenient method for preparation of nicotinuric acid (nicotinylglycine) was needed. Although one synthesis has been reported (2) for this vitamin metabolite (3), the intermediate nicotinyl chloride is troublesome to obtain and purify (4, 5).

The method developed for this work proceeds from the readily purchasable nicotinamide.



An analogy to Reaction I is found in the preparation of benzoyl hydrazide (6). Reaction II has been previously recorded (7) and Reaction III is similar to such condensations with hippuryl azide (8).

In order to effect a reaction between nicotinamide and hydrazine, it was necessary to employ concentrated solutions. 0.1 mole, 12.2 gm., of nicotinamide and 12 gm. (0.1 mole) of 42 per cent hydrazine hydrate solution were refluxed for 6 hours. The solid which separated on chilling was recrystallized from 10 cc. of aqueous ethanol (4 volumes of ethanol to 6 volumes of water). In two runs there resulted 5.9 and 7.3 gm. of nicotinyl hydrazide, melting at 158–159°.

In order to convert the hydrazide to azide, 4.1 gm. (0.03 mole) of hydrazide, 12.0 cc. (0.06 mole) of 5 N HCl, and 30 cc. of water were chilled and treated with a solution of 4.1 gm. (0.06 mole) of NaNO<sub>2</sub> in 10 cc. of water slowly, with shaking, in an ice bath. The reaction mixture was extracted with four 50 cc. portions of ether, the ether extract washed thrice with 10 per cent Na<sub>2</sub>CO<sub>3</sub> solution, twice with water, dried with CaCl<sub>2</sub>, and the ether evaporated at the water pump. A sample of the residual solid melted at 48–49°.

This was shaken with a little water and added portionwise with stirring

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and cooling to a solution of 2.25 gm. (0.03 mole) of glycine in 60 cc. of 1 N NaOH solution. This was then filtered, acidified, and immediately concentrated under reduced pressure in a water bath at 50°. When solids separated, the mixture was chilled and filtered. There was obtained 0.81 gm. melting at 238-241° with decomposition.

Recrystallization gave 0.65 gm. melting at 242-244° with decomposition. The equivalent weight by titration was 180.5 (theory, 180). A further quantity of nicotinuric acid of inferior purity could be obtained from the original mother liquor.

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# QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

## XV. THE CONCENTRATION OF SODIUM IN GLOMERULAR URINE OF NECTURI\*

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The results of a long series of experiments (1) have established the fact that the glomerular fluid of amphibia has the characteristics of an ultrafiltrate of plasma and that this fluid is modified in its passage along the tubule by reabsorption of water and solutes and by addition of some substances by the tubule cells. Although important information has been obtained concerning the acidification process from the studies of total molecular concentration (2), pH (3, 4), and ammonia (5), direct quantitative examination of the fixed bases would be of considerable value in exploring the rôle of the kidney in acid-base balance. Accordingly, beginning several years ago, work has been in progress at intervals on the determination of these elements in appropriate quantities. This paper describes an ultramicro colorimetric method for sodium and its application in a comparison of the sodium contents of serum and glomerular fluid in *Necturi*.<sup>1</sup>

### *Analytical Method<sup>2</sup>*

Sodium is precipitated as sodium zinc uranium acetate as in the Butler and Tuthill application (7) of the method of Barber and Kolthoff (8). The precipitate is washed with a solution of magnesium uranium acetate saturated with sodium magnesium uranium acetate. This reagent was used by Blanchetière (9) for the precipitation of sodium but was chosen here as a wash fluid because it is an aqueous solution free from zinc in which the precipitate of sodium zinc uranium acetate is fairly insoluble. After being washed with alcohol and ether, the precipitate is dissolved in water

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<sup>1</sup> Analyses for sodium in two samples obtained from the tubules of rat kidneys have been reported (6).

<sup>2</sup> The method may be used satisfactorily for samples of the size used in the gravimetric method and modifications could be made for samples of smaller size.

and analyzed for zinc by means of the red color developed with diphenylthiocarbazone. This colorimetric method, used by Deckert (10) for the determination of zinc in biological materials, has been applied here photo-electrically.

*Reagents—*

1. Zinc uranium acetate reagent prepared according to the directions of Butler and Tuthill (7). Several cc. of the reagent are filtered through quantitative filter paper before use.

2. Magnesium uranium acetate reagent prepared as described by Blanchetière (9). Several cc. of reagent are filtered through quantitative filter paper before use.

3. Pure 95 per cent alcohol.

4. Ether, redistilled.

5. Water, redistilled. This should be redistilled from an all-Pyrex still.

6. Sodium hydroxide, 0.01 N, carbon dioxide-free, made up in redistilled water.

7. Diphenylthiocarbazone solution, prepared immediately before use in the following manner. 100 mg. of diphenylthiocarbazone (preferably Eastman) are shaken for 3 minutes in 5 cc. of sodium hydroxide solution. This is done in a small glass-stoppered bottle or flask, the ground parts of which have been coated with a thin layer of paraffin. The excess reagent is filtered off through quantitative filter paper which has been washed in redistilled water and dried before use. If the Eastman product is used, 1 volume of the filtered reagent is diluted with 4 volumes of sodium hydroxide solution. Our experience has been that the intensity of color developed with zinc varies greatly with the brand and lot of reagent. The saturated solution of some brands may be used directly after filtration, while others will not develop sufficient color to be used in these analyses.

8. Zinc standards. Pure sodium zinc uranium acetate is prepared by precipitating the sodium of pure sodium chloride with the Butler-Tuthill reagent. 0.235 gm. of the triple salt is dissolved in redistilled water and made up to a liter. This stock solution containing 1 mg. of zinc per 100 cc. may be kept for years in a Pyrex bottle in the dark. Dilute standards containing from 10 to 70  $\gamma$  per cent of zinc are prepared frequently by diluting the stock solution with redistilled water.

9. 20 per cent trichloroacetic acid. This is made from trichloroacetic acid redistilled from an all-Pyrex still. Redistilled water is used for the solution.

*Preparation of Filters*—Filters are made by sealing partially the ends of pieces of capillary tubing and packing filter paper pulp down into the funnel ends (see Fig. 1, B). Directions are as follows: (1) Cut 3 cm. lengths of "large" capillary tubing, 0.6 mm. in internal diameter (see Richards and

coworkers (11, 12)). The ends should be cut squarely; if this is not done, the funnel openings will be off center. (2) Seal partially one end of each piece of tubing by twirling it in a micro flame, examining progress under the microscope. The opening should be funnel-shaped, and should measure approximately 0.1 mm. at the top and less at the bottom. If the opening is too large there will be a loss of precipitate, while if it is too small filtering and washing will be difficult or impossible. Discard any tubes

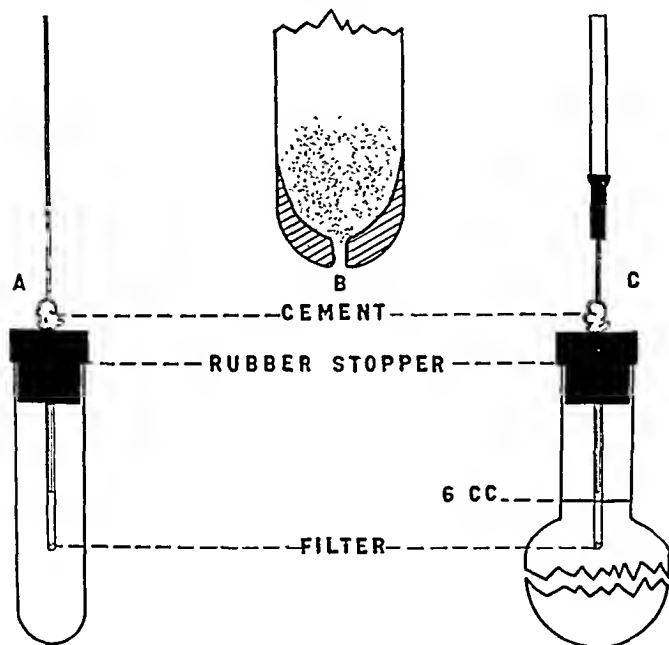


Fig. 1. Apparatus for ultramicrodetermination of sodium. A and C are approximately actual size. B is an enlargement (20 X) of the end of a filter tube.

in which the openings are of incorrect size or are off center. (3) Pack filter paper pulp down into the ends of these tubes. Prepare the pulp beforehand by teasing apart in redistilled water. Dry the pulp in an oven at 105° and use as needed. Place a small amount of the dried pulp in a Petri dish. Tease off small bits with fine forceps. Introduce these into the open ends of the filter tubes and force them down into the funnel by means of "small" capillary tubing. Pack in each bit of pulp gently but firmly using alternately the open and sealed ends of a piece of small capillary tubing about 6 cm. in length. This method produces a filter

that is well packed both in the center and on the sides. The packed filters should be 0.6 to 0.8 mm. in thickness and should show no spaces around the filter mat. Wash and test the filters as follows: By means of a syringe with an adapter attached fill the filter tubes with redistilled water. Place several of them at a time in small, short test-tubes at the bottom of which are small mats of clean, dry filter paper. Centrifuge these for about a minute and examine the tubes. Discard any which have not drained completely and place the rest in a clean weighing bottle to dry in an oven at 105°. Store the filters in the covered weighing bottle in a dust-free container.

### *Procedure for Aqueous Solutions*

*Precipitation of Triple Salt*—By means of a stage micrometer check the outside diameter of a 15 cm. length of small capillary tubing. It should measure exactly 0.5 mm. Attach the tubing to the water manipulator as described for the chloride analysis of Westfall *et al.* (12) but place the open end of the capillary over the 40 or 30 mark of the stage micrometer so that a sample of 0.2 to 0.4 c.mm. will be measured at the center of the field. The zero of the steel rule should be opposite the 35 mark on the micrometer scale. Introduce a column of sample 2 to 4 mm. in length and pull it in just far enough so that the meniscus is fully curved. Measure the sample carefully, pull it in about 5 mm., and introduce a 30 to 40 mm. column of zinc uranium acetate reagent, depending on the size of the sample. This should be done rapidly from a rather coarse capillary pipette which has just been filled with freshly filtered reagent. The measurement need not be precise but the fluid column should not be pushed back and forth, as any evaporation of the reagent would lead to high results. Pull in the reagent about 10 mm., cut off the portion of the tube containing the fluids, and seal both ends in a micro flame without heating the reagent. Centrifuge and invert the capillary ten times, allow it to stand at room temperature for 10 minutes, and then centrifuge and invert five times more. During the 10 minute waiting interval examine a filter under the microscope and repack it gently. The volume of crystals now visible at the end of the precipitation capillary will give an approximate idea of the amount of sodium in the sample. Examine the opposite end of the tube to see that no crystals have remained there. Then cut off the tip and insert the capillary into the filter tube so that the open end is about 6 mm. above the filter mat. The two tubes may now be sealed together with de Khotinsky cement as indicated in Fig. 1 without heating the reagent. Insert the capillaries into a small hole in the rubber stopper and fit this to the test-tube as shown in A. By means of rubber-tipped forceps lower the entire assembly into a centrifuge cup and spin rapidly for about 15 seconds. Lift out the assembly to examine the capillary and filter. Usually a small

amount of fluid is found above the filter at this stage. Cut off the sealed end of the capillary without disturbing the assembly, centrifuge again for about 6 seconds, and inspect. If the filter has been properly prepared, no fluid will be visible above the mat. Centrifuge for 2 minutes more to insure complete draining.

*Washing*—Place the assembly in a wooden block. Dip the end of a clean micro funnel (volume about 15 c.mm.) such as is shown at the top of *C* into magnesium uranium acetate solution. Pinch the rubber tubing attached and release it again, thus filling the funnel completely. Wipe off the outside with clean tissue and slip the rubber tubing over the end of the small capillary. Centrifuge for 6 seconds, take off the funnel, and continue centrifuging for 30 seconds more. Repeat this same process with two funnels of alcohol and two of ether. When the ether has evaporated, a layer of clean, dry, yellow crystals should be visible on top of the dry filter mat.

*Solution of Precipitate*—Transfer the stopper and capillaries to a larger tube (see *C*) calibrated to contain exactly 6 cc. to the mark. Introduce a little redistilled water into the small capillary but remove the funnel, since this entire assembly is too long for the centrifuge. Spin for a few seconds, cut the capillary off about a cm. above the de Khotinsky cement, and then proceed as before, filling the funnel with water six or seven times. This should dissolve the precipitate and carry the solution completely into the graduated tube which is then filled to the mark with water.

*Determination of Zinc*—From a 0.1 or 0.2 cc. Mohr pipette drawn out to a fine tip, add 0.1 cc. of diphenylthiocarbazone solution to each unknown tube and also to each of three colorimeter tubes containing 6 cc. of water and of two standards, respectively. Place clean corks in the unknown tubes and mix by inverting several times. Transfer the contents completely to colorimeter tubes and mix the fluids of all tubes by swirling. The blanks should be golden yellow, while the solutions containing from 10 to 70  $\gamma$  per cent of zinc vary from orange to deep cherry-red. Take readings immediately, using Filter 565 with the 6 cc. aperture of the Evelyn colorimeter. With our sample of Eastman diphenylthiocarbazone a straight line curve is obtained, the 70  $\gamma$  per cent standard reading about 34. Since there is some variation in the curve even though the solution of reagent is made up the same way each time, it has been found advisable to run at least one standard every time an unknown is run. Standards should be chosen in the range anticipated for the unknowns. Blank determinations, carried through the entire procedure with redistilled water substituted for a sample, gave maximum values of  $\pm 0.6 \gamma$  per cent of zinc. Since readings of this magnitude could be accounted for by errors in the calibration of the 6 cc. and colorimeter tubes, the blank is considered zero.

*Calculations*—Since the inside of a very curved meniscus is taken for

measurement of samples, a volume correction must be made. By inspection it was found that the addition of 0.005 c.mm. for each meniscus (0.01 in all) to the recorded volume makes the measurement sufficiently accurate for our purposes. Possibly the correction is slightly too small, but this is compensated by the fact that the inside diameter of the tube is very slightly smaller than it should be to give exactly 0.1 c.mm. per cm. of tube length. This corrected volume is used to calculate the final dilution (30,000 times in the case of a corrected sample volume of 0.2 c.mm.) and

TABLE I  
*Results of Single Ultramicroanalyses for Sodium (NaCl Solutions)*

Volume of sample	Sodium in sample	Concentration	Concentration found	Per cent error
c.mm.	$\gamma$	mg. per cent	mg. per cent	
0.403	1.26	314	303	-3.5
0.405	0.815	201	198	-1.5
0.221	0.694	314	303	-3.5
0.208	0.490	236	238	+0.9
0.410	0.435	106	108	+1.9
0.212	0.426	201	198	-1.5
0.208	0.360	173	178	+2.9
0.210	0.346	165	154	-6.7
0.193	0.274	142	150	+5.6
0.203	0.216	106	117	+10.4
0.200	0.190	95	105	+10.5

since there is 1 atom of sodium for each of zinc in the triple salt, calculations are as follows:

$$\text{Concentration of Na in sample} = \frac{\text{atomic weight of Na, 23.0}}{\text{atomic weight of Zn, 65.38}}$$

$$\times \text{concentration of Zn in final solution} \times \text{dilution}$$

*Results on Aqueous Solutions*—In 1938 a series of eight and in 1941 another of eleven preliminary determinations were made. In these the sodium content of samples was from 0.2 to 0.7  $\gamma$ , the average error was 4.6, and the maximum error 13 per cent. In the past year the method has been modified slightly and reexamined for accuracy. Results of analyses made during the past year with the technique described above are shown in Table I. The composition of these solutions was unknown to the analyst. When samples contained 0.3 to 1.3  $\gamma$  of sodium, the maximum error was  $\pm 7$  per cent, but the usual error was considerably less. If still smaller amounts were used, the error was increased, as shown in Table I. In all the animal experiments to be described the samples contained at least 0.3  $\gamma$ .

*Analysis of Serum*—Directions for serum are as follows: Draw into a large capillary a column of serum 3.0 mm. in length and follow this with a column of equal length of trichloroacetic acid. Seal the tube and mix

TABLE II

*Comparison of Results Obtained on Serum with Gravimetric and Ultramicromethods*

Type of serum	Gravimetric	Ultramicro	Difference
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
Dog	339	350	3.2
<i>Necturus</i> , pooled	220	218	0.9
" "	217	220	1.4
" "	227	222	2.2
" "	233	236	1.3
" "	217	236	8.8
Average.....			3.0

TABLE III

*Sodium Concentration of Blood Serum and Glomerular Fluid of Necturus*

Date	Rate of fluid collection	Sodium concentration				Difference
		Serum I	Serum II	Average	Glomerular fluid	
1942	<i>c.mm. per hr.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>per cent</i>
Jan. 5.....	1.2	246	262	254	227	-10.6
Feb. 23.....		220	188	204	203	+2.0
Mar. 3.....	0.5		259		226	-12.7
" 9.....	1.0	270	286	278	273	-1.8
" 16.....	1.0	256	242	249	242	-2.8
Apr. 10.....	0.5	226	229	227	202	-11.0
" 17.....	0.5	216	218	217	186	-14.3
May 15.....	0.7	266	256	261	267	+2.3
June 19.....	0.4	233	196	214	203	-5.1
" 29.....	1.5	236	236	236	256	+8.5
July 1.....	0.8	215	213	214	224	+4.7
" 7.....	0.3	241	244	242	230	-5.0
" 9.....	0.2	254	235	244	266	+9.0
" 21.....	0.4	264	248	256	274	+7.0
" 24.....	1.2	274	261	267	278	+4.1
Mean.....						-1.7

the contents by three centrifugations; allow the mixture to stand for 10 minutes, and then centrifuge again for 2 minutes. Use approximately 0.4 c.mm. for sodium analysis as described before. In Table II is shown a comparison of results obtained on serum by gravimetric and ultramicro-



methods. In most cases the gravimetric analyses were carried out in duplicate, while the ultramicrodeterminations were done singly. Agreement was good in most cases. In the last example in which the difference was 8.8 per cent the ultramicroanalysis was repeated twice, the results being 232 and 236 mg. per cent. Unfortunately there was insufficient serum to repeat the gravimetric analysis.

### *Animal Experiments*

The glomerular puncture technique of Wearn and Richards (13) was used on *Necturi* as described by Walker and Reisinger (14). Blood was taken from the posterior cava before and after the glomerular fluid collection and allowed to clot in capillaries before centrifuging. The results of fifteen experiments are shown in Table III. In nine of the experiments the difference between the average serum sodium and that of glomerular fluid is approximately 5 per cent or less. The greatest differences are -14 and +9 per cent. It is believed that these differences are within the experimental error of the entire procedure and that the sodium content of the two fluids is essentially the same. The range of protein concentration which White (15) found for *Necturus* serum was 1.2 to 3.5 per cent. Analyses in two of our animals also showed serum protein in this range. The sodium content of ultrafiltrates of such plasmas would be close to that of the plasma itself, though probably slightly lower. It may be a coincidence or a reality that the mean difference in these experiments is slightly negative.

### SUMMARY

1. A colorimetric ultramicromethod for the determination of sodium is described. The principle of the method is the precipitation of sodium as sodium zinc uranium acetate and the determination of zinc in a solution of the salt by means of the delicate color reaction with diphenylthiocarbazone. Samples as small as 0.2 c.mm. and containing as little as 0.3  $\gamma$  of sodium are determined with an average error of approximately 3.0 per cent.

2. In nine out of fifteen experiments on *Necturi* the sodium content of glomerular fluid was within 5 per cent of that of serum. The greatest differences were -14 and +9 per cent. These results are interpreted as adding another link to the long chain of evidence characterizing the glomerular fluid as an ultrafiltrate of plasma.

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# THE SULFUR DISTRIBUTION IN THE RIB-GRASS STRAIN OF TOBACCO MOSAIC VIRUS

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The differences in biological properties which distinguish various strains of a virus are presumably accompanied by corresponding variations in structure, in chemical composition, or in both. Analyses of highly purified preparations of several strains of tobacco mosaic virus indicate that these viruses are nucleoproteins (1). The particle shapes and sizes of these nucleoproteins are practically indistinguishable in the analytical ultracentrifuge and in the electron microscope (1).<sup>1</sup> Moreover, the x-ray patterns of five strains indicate that they possess similar gross internal structures and similar subunits (2). Hence it might be concluded that the chemical differences between strains are not great, although at least one example to the contrary is now known (3-5). Chemical differences may lie in the nucleic acid portion or in the protein component of the virus molecules. Analyses carried out on eight different strains of tobacco mosaic virus have shown that these particular strains contain equivalent amounts of ribose nucleic acid but that their protein components possess marked differences in amino acid composition (3, 4). Despite the fact that analyses have been made for only five amino acids, differences have been found in the compositions of six of the eight strains. The composition of the rib-grass strain differs most widely from that of the ordinary strain, for, in addition to containing amounts of tyrosine, tryptophane, and phenylalanine which are considerably different from those of the type strain, the rib-grass virus contains 0.55 per cent histidine, an amino acid entirely lacking in the type strain and in six other strains (4). Moreover, the rib-grass strain contains about 3 times as much sulfur as the ordinary strain (5). The results of an investigation on the nature of this comparatively large amount of sulfur are given in the present communication. Since the 0.2 per cent of sulfur in ordinary tobacco mosaic virus has been accounted for as cysteine (6-8), it was of special interest to determine whether the increased amount of sulfur in the rib-grass strain is all present as cysteine or whether some other sulfur-containing amino acid likewise occurs in this protein.

<sup>1</sup> Knight, C. A., and Stanley, W. M., unpublished data.

## EXPERIMENTAL

The rib-grass virus was obtained from diseased Turkish tobacco plants as recently described (5). Highly purified preparations of virus were frozen and dried *in vacuo* and then further dried to constant weight in an oven at 110°. The white fluffy material secured was used for most of the analyses. Aqueous solutions of highly purified virus were used for the remainder. Four total sulfur determinations made by commercial analysts yielded values ranging from 0.52 to 0.69 per cent, with an average of 0.62 per cent. At least a portion of this sulfur was assumed to be in the form of cysteine, since even mildly denatured virus gave a strongly positive nitroprusside test. An indication that part of the sulfur was present as methionine was obtained by means of the color test developed by Kolb and Toennies (9). Of eight strains of tobacco mosaic virus tested, only the rib-grass strain gave a positive reaction.

TABLE I  
*Distribution of Sulfur in Rib-Grass Virus*

Sample No.	Cysteine			Methionine			Sulfate sulfur
	Ferricyanide method	Uric acid method	Baernstein titration	Volatile iodide	Homo-cysteine	Nitroprusside method	
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	0.68	0.68					
2	0.68	0.70					
3		0.69					
4		0.70					
5		0.73					
6			0.55	2.0	1.9		0
7			0.60	2.1	2.1	1.9	0

*Cysteine and Methionine*—Cysteine was determined in solutions of the virus by direct titration of the —SH groups with standard ferricyanide in the presence of guanidine hydrochloride (10, 8), and by a colorimetric method involving the reduction of Folin's uric acid reagent by the virus in the presence of urea (11). In addition, some values for cysteine, methionine, and sulfate sulfur were obtained by the Baernstein method as modified by Kassell and Brand (12). One of the samples examined by the Baernstein method was also analyzed for methionine by the colorimetric procedure of McCarthy and Sullivan (13). The results of the analyses are summarized in Table I.

## DISCUSSION

The cysteine value obtained by the Baernstein method was definitely lower than those obtained by the uric acid and ferricyanide determinations.

This can probably be attributed at least in part to the method, since it is known to yield low values with pure cysteine (6, 12). No correction factor was used to compensate for this fact. The value of 0.58 per cent cysteine in the rib-grass strain corresponds quite closely to the uncorrected average of 0.61 per cent obtained by the Baernstein method by Ross for the ordinary strain (6). In addition, neither the ferricyanide titration nor the uric acid method as applied in the present experiments revealed any significant difference between the rib-grass virus and ordinary tobacco mosaic virus. The latter is considered to contain 0.68 per cent of cysteine (6-8). These results suggest that the two strains contain nearly identical amounts of cysteine.

Since no sulfate sulfur was found in the rib-grass virus, it was assumed that the sulfur of this virus is distributed between the amino acids, cysteine and methionine. A combination of the cysteine and methionine sulfur values of the Baernstein analysis yields a total of 0.58 per cent sulfur. Similarly, a combination of the average cysteine sulfur indicated by the ferricyanide and uric acid methods with the McCarthy and Sullivan methionine sulfur gives a total of 0.59 per cent sulfur. These values agree well with the average figure of 0.62 per cent sulfur obtained by elementary analyses. It may be concluded, therefore, that all or essentially all of the sulfur of the rib-grass virus has been accounted for in the forms of cysteine and methionine.

The 2 per cent of methionine found in the rib-grass virus represents another striking difference between this strain and seven other strains of tobacco mosaic virus, for these appear to contain no methionine. While the rib-grass virus closely resembles the ordinary strain in size and shape, in immunological, serological, and gross chemical and physical properties, it differs distinctly from the latter in certain biological properties and chemically in at least five important respects. It is hoped that a correlation may shortly be made between some of the chemical and biological differences.

#### SUMMARY

Analysis of the rib-grass strain of tobacco mosaic virus indicated that this virus contains about 0.62 per cent of sulfur, or about 3 times the proportion found in ordinary tobacco mosaic virus. The cysteine content of the rib-grass virus, as measured by three different methods of analysis, is apparently the same as in the ordinary strain; *i.e.*, about 0.68 per cent. However, quantitative analyses by two different methods indicated the presence of about 2 per cent of methionine and qualitative tests showed that the rib-grass virus is the only one of eight strains which contains methionine. All or essentially all of the sulfur of the rib-grass virus has been accounted for in the cysteine and methionine which the virus contains.

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## STUDIES OF PROTOPORPHYRIN

### I. THE PURIFICATION OF PROTOPORPHYRIN IX AS OBTAINED FROM HEMOGLOBIN\*

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Protoporphyrin is easily prepared from hemoglobin according to the method of Fischer and Pützer (1). The purification of the crude protoporphyrin, and especially the removal of a brown-green substance which interferes with crystallization of the protoporphyrin or its methyl ester, is relatively difficult. We have frequently found it impossible to crystallize the free protoporphyrin or its methyl ester by means of methods hitherto described (1-3). Hamsik (2) briefly referred to a method for crystallizing free protoporphyrin, which we have found both simple and efficient. Hamsik (2) evidently regarded this only as a means of recrystallizing material that was already relatively pure, but in our experience the method is applicable to the crude protoporphyrin as first obtained with the Fischer-Pützer method. This consists in dissolving the dry porphyrin in a small amount of pyridine and then adding petroleum ether (b.p. 30-60°) to the point of beginning precipitation. The precipitate is crystalline, exhibiting the habitus described by Fischer, Treibs, and Zeile (3). The crystallization is nearly quantitative when sufficient petroleum ether is added and the mixture allowed to stand in the ice box. After four recrystallizations, microanalysis<sup>1</sup> for C and H yielded the following results.

$C_{54}H_{54}N_4O_4$ . Calculated, C 72.56, H 6.09; found, C 71.32, H 5.94

The direct crystallization of the crude methyl ester of protoporphyrin IX from chloroform-methyl alcohol or benzene-petroleum ether (4) is unsatisfactory. In a previous study by Schwartz and Watson (5) it was found that the protoporphyrin methyl ester obtained from feces was readily purified by the chromatographic method, with Brockmann's  $Al_2O_3$  (Merck), and  $CHCl_3$ -petroleum ether as eluent. The method is as follows:

\* Aided by a grant from the Medical Research Fund of the Graduate School, University of Minnesota.

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<sup>1</sup> This was carried out in the micro laboratory of the Division of Organic Chemistry, University of Minnesota.



The ester dissolved in chloroform is purified in the usual way by washing with distilled water, 10 per cent ammonia, and 7 per cent sodium chloride solution. The solution is dried by filtering through  $\text{CHCl}_3$ -moistened paper, after which it is concentrated to a small volume on the water bath and mixed with 12 volumes of petroleum ether; precipitation of the majority of the brown-green impurity together with some of the protoporphyrin ester takes place. After a few minutes the precipitate is separated by filtration and the filtrate is passed through a column of  $\text{Al}_2\text{O}_3$  (we started with Brockmann's  $\text{Al}_2\text{O}_3$ , but have found subsequently that the product of the Aluminum Ore Company (mesh No. 100-120) gives the same results).<sup>2</sup> The process can be accelerated by applying a little suction but the chromatogram is more perfect and the purification is better without suction. The precipitate from the above filtration is redissolved in a small volume of chloroform which is then mixed with 10 volumes of petroleum ether and filtered as before. This filtrate is also passed through the same column of  $\text{Al}_2\text{O}_3$  at a time when the first filtrate has almost completely gone through. This operation is repeated with increasing concentrations of the chloroform in the petroleum ether, 1:8, 1:6, 1:4, 1:2, 1:1, and finally with chloroform alone. The adsorption of the entire amount of porphyrin and the impurities on the top of the column takes place as a rule during the passage of the first two fractions. With the fraction at a concentration of 1:8 the chromatogram begins to develop. A zone of the protoporphyrin ester, representing the purest fraction, starts to wander down the column. This is eluted as the increasing concentrations of  $\text{CHCl}_3$  in petroleum ether are passed through. At the same time a development of more zones occurs, representing the ester with increasing impurities. Finally several portions have been collected, the first the purest and the last the most impure. Most of the brown-green substance remains adsorbed on top of the column. The first fractions crystallize very easily, often spontaneously from the chloroform-petroleum ether during the 1st hour or on standing overnight. If a greater yield is desired, those fractions which do not crystallize well combined with the mother liquors of the crystals are dried in a water bath, after which the residue is redissolved in  $\text{CHCl}_3$  and petroleum ether and subjected anew to the chromatographic procedure. After two recrystallizations from  $\text{CHCl}_3$ -methyl alcohol, the melting point of the crystals was  $223\text{--}224^\circ$  (not corrected). This material was a chromatographic entity, as is shown by

<sup>2</sup> Recently we have found that precipitated calcium carbonate (Merek or Cenco, U.S.P.) may be used to advantage when the original Tswett procedure with Zecheimer-Cholnoky tubes (6) is used. Adsorption is effected from chloroform-petroleum ether, 1:10, and the chromatogram is developed with chloroform-petroleum ether, 1:4.

redissolving it in  $\text{CHCl}_3$ , mixing with petroleum ether, and passing through  $\text{Al}_2\text{O}_3$  as described above. Development with a 1:6 mixture resulted in the appearance of but one zone, leaving the column above it completely white, without pigment. The ester thus obtained is undoubtedly a purer preparation than that of the free protoporphyrin.

The microanalysis for C and H, on this material, was as follows:

$\text{C}_{33}\text{H}_{33}\text{N}_4\text{O}_4$ . Calculated, C 73.19, H 6.49; found, C 72.66, H 6.25

*Saponification of Ester*—The ester is best saponified by allowing it to stand overnight in the refrigerator, in 25 per cent HCl. This is simpler and less destructive than the method in which sodium hydroxide and heat are employed. The saponification is usually complete after 5 to 6 hours.

#### SUMMARY

Improved methods of purifying protoporphyrin and its methyl ester, as prepared from hemoglobin, are described.

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## STUDIES OF PROTOPORPHYRIN

### II. A NOTE ON AN IMPROVED MICROMETHOD FOR CONVERTING PROTOPORPHYRIN TO MESOPORPHYRIN\*

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(Received for publication, October 10, 1942)

Fischer and Kögl (1) were the first to recognize that small amounts of protoporphyrin are often difficult to purify sufficiently for identification, and that it is therefore best to convert the protoporphyrin to mesoporphyrin which is easily handled and whose isomer type may be identified with ease by means of melting point determination. Various micromethods for this conversion have been described (1-3). The improved procedure recently described by Schultze (3) increased the yield from the 10 to 20 per cent obtained with the original Fischer-Kögl method to 35 to 40 per cent. In brief, the method of Schultze is as follows:

To 10 mg. of protoporphyrin dimethyl ester are added 100 mg. of ascorbic acid and 2 ml. of glacial acetic acid. 0.3 ml. of HI (sp. gr. 1.94) is then added, and the mixture is boiled for 45 seconds. After cooling rapidly, the solution is mixed with 50 ml. of ether, which is then washed twice with 15 ml. portions of a 10 per cent aqueous solution of sodium acetate and 3 per cent sodium sulfite. The ether solution is next washed with water, and the mesoporphyrin is extracted with small amounts of 5 per cent hydrochloric acid. The extract is neutralized with sodium hydroxide and sodium acetate, and the porphyrin is then extracted with ether. From the ethereal solution the porphyrin is removed with 2.5 per cent HCl. The HCl solution is then washed with a mixture of 2 parts of ether and 1 part of chloroform. The porphyrin is precipitated from the acid solution by the addition of sodium hydroxide. The insoluble sodium salt is collected by centrifuging. After repeated washing with water, it is directly esterified with methyl alcohol saturated in the cold with HCl gas, and the ester is then separated and crystallized from chloroform-methyl alcohol in the usual way (1). As already noted, the yield with this procedure is 35 to 40 per cent of the original amount of protoporphyrin. With Rimington's modification (2) of the Fischer-Kögl method we have obtained a yield of approximately 30 per cent.

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In working with the earlier methods of Fischer and Kögl as well as the later one of Schultze, just described, we find that it is unnecessary to use hydriodic acid of specific gravity 1.94. Hydriodic acid with a specific gravity of 1.50, which is much easier to obtain, is just as efficient if used in somewhat larger amounts.

Two sources of loss have been noted in Schultze's method. The first is that an appreciable amount of porphyrin is removed in washing the first ether with the sodium acetate-sodium sulfite mixture. The material must in turn be washed with ether if this loss is to be avoided. A second source of loss is that a considerable fraction of mesoporphyrin is often not removed from the ether with 5 per cent or even with 10 per cent HCl. Furthermore, a large loss occurs when the HCl solution is washed with the mixture of ether and chloroform.

With these facts in mind, the following modified procedure has been worked out with a resultant yield of approximately 60 per cent.

### *Method*

To 10 mg. of protoporphyrin dimethyl ester are added 100 mg. of ascorbic acid, 2 ml. of glacial acetic acid, and 0.6 ml. of HI (sp. gr. 1.50). The mixture is boiled for 45 seconds. After cooling rapidly, the solution is at once mixed with 50 ml. of ethyl ether (peroxide-free); any undissolved porphyrin at this stage is dissolved in a little glacial acetic acid and added to the ether. The ether solution is then washed twice with the sodium acetate-sodium sulfite solution (see above) and three times with distilled water. The combined washing solutions are extracted repeatedly with ether. The product is combined with the main ether extract from which the porphyrin is then removed by repeated extraction with 5 per cent HCl. Occasionally a small amount of porphyrin together with a yellowish brown impurity remains in the ether; the mesoporphyrin is not adequately extracted unless 25 per cent HCl is used. A separate extraction with this concentration is therefore carried out. The 25 per cent HCl extract is worked up separately in the same manner as is the 5 per cent. It would probably be satisfactory in most instances to extract directly with 25 per cent HCl, but since this removes more impurities we have always used 5 per cent first, with the thought that, in any instance in which the amount of mesoporphyrin was relatively small and the amount of impurities large, crystallization might not be achieved at all if only a 25 per cent concentration was used. Except when it is desired to increase the yield to the utmost, the 25 per cent extraction may be omitted. The HCl extracts are neutralized with sodium acetate and the mesoporphyrin is extracted with ether. The ether is washed three times with distilled water (the combined wash water in turn being extracted with a few ml. of ether

which are added to the main ethereal extract). The ether is then extracted with 2.5 per cent HCl which now removes the mesoporphyrin almost quantitatively. The HCl extract is neutralized with sufficient 20 per cent NaOH to make the solution negative to Congo red. The mesoporphyrin largely precipitates after a few hours and is then collected on a small filter paper.<sup>1</sup> After being washed with small amounts of distilled water, the precipitate is dried and esterified by solution in methyl alcohol saturated in the cold with HCl gas. After standing overnight, the ester is crystallized in the usual way from  $\text{CHCl}_3$ -methyl alcohol. After two to three recrystallizations a melting point of  $212\text{--}214^\circ$  (not corrected) is observed. The yields shown in Table I have been obtained.

In Table I no account was taken of crystals left on the sides of the tube or remaining in the mother liquors of the recrystallizations. It may be

TABLE I

*Yields of Mesoporphyrin Ester Obtained from Varying Amounts of Crystalline Protoporphyrin Ester*

Experiment No.	Protoporphyrin methyl ester	Mesoporphyrin ester obtained	Yield
	mg.	mg.	per cent
1	9.5	5.8	61
2	5.5	3.6	65
3	10.0	5.9	59

noted, however, that in Experiment 3, 1.5 mg. of the total yield were recovered from an ether-chloroform mixture (2:1) which had been used to wash the 2.5 per cent HCl extract, according to Schultze's procedure. This step was not included in Experiments 1 and 2.

## SUMMARY

An improved micromethod is described for the conversion of proto- to mesoporphyrin. The yield with this method is approximately 60 per cent.

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<sup>1</sup> Small portions remaining in solution are recovered by ether extraction, after which the ether is concentrated to dryness and the residue is esterified.



## STUDIES OF PROTOPORPHYRIN

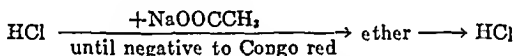
### III. PHOTOELECTRIC AND FLUOROPHOTOMETRIC METHODS FOR THE QUANTITATIVE DETERMINATION OF THE PROTOPORPHYRIN IN BLOOD\*

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(Received for publication, October 10, 1942)

The presence of free protoporphyrin in human erythrocytes was reported in 1928 by Hijmans van den Bergh and Hyman (1) who devised a method, further modified by van den Bergh and Grotepass (2), for its quantitative determination. The method entails extraction of the red cells separated from 30 to 40 ml. of blood with 100 to 150 ml. of a mixture of ethyl acetate and glacial acetic acid (3:1). This mixture is washed with distilled water and the protoporphyrin is extracted with 5 per cent HCl. After one purification



the porphyrin content of the final HCl extract is determined on the basis of the intensity of its red fluorescence. Modifications of this procedure have been described by Lageder (3), Vigliani and Angeleri (4), Schumm (5), and Seggel (6). In all of these, the principle of extraction of the protoporphyrin is the same as in the original van den Bergh-Grotepass method. The chief difference between them is in degree of purification of the final HCl extract and in the physical method of estimation of the porphyrin. In some, the concentrations are measured by means of the intensity of the red fluorescence, in others by means of the intensity of absorption of ultraviolet light (Schumm) or of visible light (Seggel). All of these methods have obvious limitations. Those based on the intensity of red fluorescence are subject to the difficulty that the amounts are often small and the intensity correspondingly weak. Under these circumstances a color match in the stufenphotometer, as in Lageder's method (3), is often impossible. Furthermore, as Lageder points out and we can confirm, protoporphyrin solutions are not particularly stable when exposed for any long period to ultraviolet light, as is often necessary in attempting to obtain a color match

\* Aided by a grant from the Medical Research Fund of the Graduate School, University of Minnesota.

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with the stufenphotometer. As regards Seggel's method, our experience indicates that the amounts of porphyrin are rarely large enough to permit any accuracy of measurement of absorption in the visible spectrum, unless very large amounts of blood are worked up. We find no objection to the spectrographic method of Selummm other than the relative inaccessibility of the apparatus and greater technical difficulty and the expense of employing it. The measurement of the much greater absorption in the near ultraviolet region, according to Selummm, is clearly advantageous and is embodied in the method to be described.

#### EXPERIMENTAL

Earlier studies<sup>1</sup> relative to the determination of small amounts of protoporphyrin were carried out with the Zeiss stufenphotometer. These will be mentioned now only because they reveal certain characteristics of protoporphyrin which it is wished to record. The source of ultraviolet light was a small, high pressure mercury arc lamp (Mico type) firmly attached to the front of the photometer. The light was filtered through a heat-resisting, red-purple ultra filter, Corning No. 587. The shelf for the cells between the filter and the photometer was cooled by a small electric fan. A red fluorescent glass standard (Zeiss) of the type used by Lagerder (3) was employed in place of a standard solution. By recording the fluorescence of this glass standard photographically, with a small spectrograph, it was possible to determine that no measurable change in intensity occurred after periods totaling about 40 hours of continuous irradiation at temperatures varying up to 50°.

The Zeiss No. L-1 red filter was interposed in the common path of the light beams from the two sources. With the stufenphotometer, it was found that the intensity of red fluorescence of protoporphyrin diminishes with increasing concentration of HCl (from 1 to 5 per cent). This is shown in Fig. 1. Even in 1 per cent HCl, however, the fluorescent intensity is too small for accurate measurement below 40  $\gamma$  per cent. The data are shown in Fig. 2. In addition, it was found that the fluorescence diminishes with increasing temperature and with continuous ultraviolet irradiation. These data need not be given, since the stufenphotometer is not used in the method described below.

The above experiments indicated clearly that the stufenphotometer was not sufficiently sensitive for the small amounts of protoporphyrin present in the erythrocytes, especially when it was considered that the maximum fluorescence was in 1 per cent HCl, and that efficient extraction of protoporphyrin from ether requires at least 2 per cent HCl.

At the time that the above preliminary studies were completed, a more

<sup>1</sup> These were carried out with Dr. Irwin Vigness of the Division of Biophysics.

sensitive fluorophotometer was not available. We determined, therefore, to measure the absorption at  $407\text{ m}\mu$  as Schumm (5) did spectrographically, but to use instead the Evelyn photoelectric colorimeter. The following method was devised and has now been in use for some time with entirely satisfactory results.

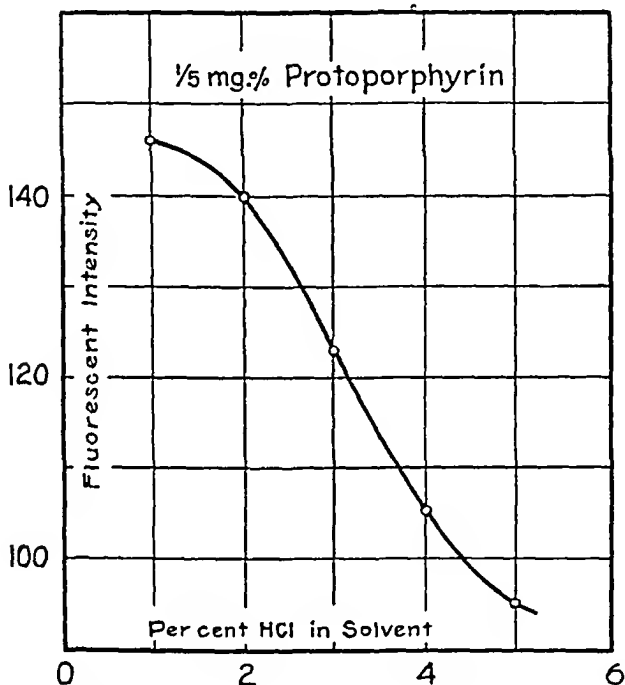


FIG. 1. Diminishing intensity of fluorescence with increasing concentration of HCl (stufenphotometer).

At least 5 ml. of red blood cells are washed twice with physiological saline, after which they are well homogenized with 50 ml. of a mixture of ethyl acetate and glacial acetic acid (3:1). This operation is carried out by means of a glass rod in a large Pyrex test-tube, preferably with a lip (30 × 100 mm.). This mixture is then quantitatively transferred to a 150 ml. Pyrex glass-stoppered bottle. It is shaken by hand for 1 to 2 minutes, then allowed to stand for 10 minutes, after which the supernatant fluid is decanted through a filter paper. The residue is washed three times with 15 ml. portions of the 3:1 mixture of ethyl acetate-glacial acetic acid. The

filtrate is placed in a small separatory funnel and washed three times with distilled water. The combined water washings are extracted with 10 ml. of ethyl acetate to recover any protoporphyrin present, and this is added to the main ethyl acetate fraction. After complete separation of the water, the ethyl acetate is extracted with 2 ml. portions of 10 per cent HCl

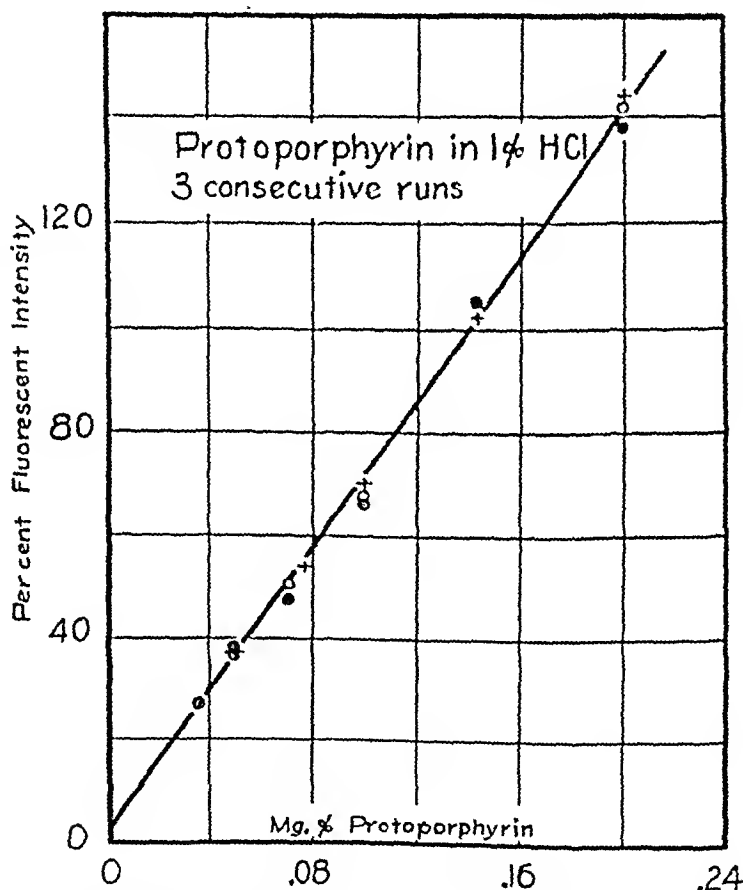


FIG. 2. Fluorescent intensity of varying concentrations of protoporphyrin in 1 per cent HCl (stufenphotometer).

until no further fluorescence is observed. For this purpose a carbon arc lamp fitted with a Corning No. 587 filter is used. The combined HCl extract is neutralized with sodium acetate until negative to Congo red paper. 1 ml. of glacial acetic acid is added. This reduces the tendency to emulsion in the subsequent extraction. The solution is then extracted three times with equal volumes of ethyl ether. The combined ether extract is

washed once with a few ml. of 1 per cent sodium carbonate (not enough to neutralize the acetic acid in the ether completely). Next the ether is washed twice with water. Finally the ether is extracted repeatedly with 1.5 ml. portions of 5 per cent HCl until fluorescence is no longer observed, which usually requires three to four extractions. The HCl extracts are collected in a graduated glass-stoppered 10 ml. cylinder and made up to 10 ml. with 5 per cent HCl. The solution is well mixed and the absorption is then measured in the Evelyn colorimeter as noted below. The concentration may be calculated according to the formula  $(R/V) \times 10 =$  micrograms of protoporphyrin in 100 ml. of cells, where  $R$  = the value obtained from the calibration curve as given below, and  $V$  = the volume of red blood cells used.

The method is equally satisfactory for whole blood, but will, of course, include any porphyrin present in the plasma. The present status of the

TABLE I

*Data for Construction of Calibration Curve for Determining Protoporphyrin with Evelyn Photoelectric Colorimeter*

Protoporphyrin <i>γ per cent</i>	Galvanometer reading (Evelyn colorimeter)
11	89 <sup>1</sup>
33	72
55	60
77	51 <sup>2</sup>
110	41 <sup>3</sup>
132	37

plasma porphyrin is not clear. There is general agreement that the normal plasma is free of porphyrin (2, 5). The occurrence of coproporphyrin in the plasma in various diseases was reported by van den Bergh and co-workers (7), and by Vigliani and Angeleri (4). More recently, however, Schumm (5) reported the occurrence of protoporphyrin in the plasma, albeit in different types of cases than those studied by the previous workers. This problem obviously merits further investigation. In the meanwhile, it is undoubtedly best to apply the present method to the red cells only.

As Schumm (5) has pointed out, the maximum absorption of protoporphyrin dissolved in 5 per cent HCl is at 407.4 mμ. This absorption is much more intense than any in the visible spectrum.

The calibration curve was prepared with pure protoporphyrin obtained from hemoglobin according to Fischer and Pützer (8) and purified as described in Paper I of this series. The crystalline ester was weighed on a micro balance after which it was saponified in 25 per cent HCl and was then

diluted with water to 5 per cent. The absorption of a series of dilutions was measured with the No. 400 filter. Table I includes sufficient data for the construction of the calibration curve.

The absorption of the final HCl solution is constant for from 6 to 24 hours. After 24 hours there is always a varying but moderate decrease, rarely more than 15 per cent of the original value over long periods (Table

TABLE II  
*Stability of Protoporphyrin (Absorption) in Final HCl Extract As Measured by Evelyn Photoelectric Colorimeter*

Experiment No.	Measured immediately	Measured after 24 hrs.	Measured after 48 hrs.
1	13.4	11.5	
2	18.2	15.5	
3	13.5	11.5	11.0
4	15.0	14.0	13.7
5	10.0	15.5	15.2
6	21.5	18.0	17.4
7	15.0	15.0	
8	17.2	17.2	
9	14.0	14.0	
10	12.5	11.5	11.0
11	10.5	8.8	8.8

TABLE III  
*Stability of Protoporphyrin (Fluorescence) in Final HCl Extract As Measured by Coleman Fluorophotometer*

Experiment No.	Measured immediately	Measured after 24 hrs.
1	13.2	5.8
2	18.7	7.4
3	18.0	7.1
4	15.0	6.0
5	16.0	7.5
6	12.0	4.5
7	18.2	8.0

II). On the contrary, measurement of the concentration by means of the fluorophotometer (see below) has shown, in agreement with the findings of Lageder (3), a marked decrease in the intensity of the fluorescence on standing; after 24 hours the decrease is more than 50 per cent even when the solution is kept in the dark (Table III). It is evident that the protoporphyrin undergoes some molecular change in which the absorption at 400  $m\mu$  remains practically the same, while the fluorescence diminishes mark-

edly. Solutions of pure protoporphyrin are much more stable; we have noted no deterioration of absorption with the Evelyn colorimeter within 3 days, nor of fluorescence, with the fluorophotometer (see below) within 24 hours. These, of course, were subjected to ultraviolet light only long enough to obtain a reading, which is a matter of but a few seconds with the fluorophotometer.

*Specificity of Absorption*—In order to be sure that the absorption at 400  $m\mu$  in the Evelyn colorimeter is due only to protoporphyrin, we compared the results obtained with the Evelyn instrument with those obtained by measuring the intensity of fluorescence with the fluorophotometer (Cole-

TABLE IV

*Comparison of Fluorescence and Absorption Intensities of Final Protoporphyrin Solutions from Various Samples of Blood*

Experiment No.	Concentration of protoporphyrin, $\gamma$ per 100 ml. blood	
	Evelyn photoelectric colorimeter	Coleman fluorophotometer
1	11.0	11.0
2	10.5	11.0
3	9.2	9.2
4	31.7	32.1
5	10.0	9.2
6	13.4	13.2
7	12.9	13.2
8	18.2	18.7
9	17.0	18.0
10	13.0	13.0
11	12.0	12.0
12	18.5	18.2
13	10.0	10.0
14	93.3	91.1

man) with the lamp filter No. B-2 and the photocell filter No. PC-2 but without the No. 032 amber filter. The photocell in this apparatus is not as sensitive in the red<sup>2</sup> as in the yellow. Nevertheless, the arrangement was satisfactory for the purpose desired, since we simply wished to obtain a series of comparative values between absorption and fluorescence. If these values had been found to differ significantly, it might have been assumed that interfering compounds were present. This was not the case, however, as proved by the agreement of the data presented in Table IV.

The data used in constructing the fluorophotometric curve are given in

<sup>2</sup> Recently we have found the Klett fluorophotometer to be much more sensitive in the red and correspondingly more satisfactory.

Table V. To obtain them, pure solutions were employed whose concentration was determined by means of the Evelyn colorimeter, as described above. The relative sensitivity of the Coleman fluorophotometer was fixed with a standard solution of quinine sulfate containing 0.3 mg. per liter in 0.1 N sulfonic acid. We adjusted the sensitivity of the apparatus to a galvanometer deflection of 96, using the quinine solution and the filters mentioned above. In calculating the amount of protoporphyrin in the

TABLE V

*Data for Construction of Calibration Curve for Determining Protoporphyrin for Coleman Fluorophotometer*

Protoporphyrin <i>γ</i> per cent	Galvanometer reading (Coleman fluorophotometer)
5.0	5.0
13.5	12.0
23.0	19.0
35.0	27.0
50.0	34.5
60.0	38.3
73.0	42.8

TABLE VI

*Comparison of Effect on Protoporphyrin Content of Brief and Prolonged Shaking during Primary Extraction of Red Blood Cells*

Experiment No.	Protoporphyrin	
	Shaking 2 min. <i>γ</i> per cent	Shaking 2 hrs. <i>γ</i> per cent
1	31.2	30.0
2	38.0	35.0
3	41.0	40.0
4	32.0	27.5
5	37.5	32.3
6	13.0	12.0

red cells, the same formula was used as is given above for the Evelyn photoelectric colorimeter.

In the method described above, the preliminary treatment leading up to the colorimetric determination has been altered from that of the original van den Bergh-Grotepass procedure with respect to the time of shaking the blood and ethyl acetate-glacial acetic acid mixture. The reason for this change is as follows:

In van den Bergh's method the mixture is shaken for 2 hours. As

shown in Table VI, this is unnecessary and in fact undesirable; better yields are obtained with only brief shaking; during the prolonged shaking some of the protoporphyrin is probably destroyed.

The purification of the first HCl extract, as in the method of van den Bergh and Grotepass, has been retained for the following reasons. The first HCl extract contains traces of hemin and some opalescence; the amount of both varies from one determination to another. The opalescence in the HCl extract increases when the ethyl acetate-glacial acetic acid filtrate is not extracted immediately. Both hemin and opalescence cause an increased absorption at 400 m $\mu$ . In accordance with van den Bergh's procedure we therefore purify further by taking the porphyrin into ethyl ether and then extracting it with 5 per cent HCl, thus obtaining a clear and colorless solution. Seggel (6) states that a second extraction entails a considerable loss of porphyrin. We have found, however, that

TABLE VII

*Recovery of Protoporphyrin Added to First HCl Extract (10 Cc. Volume)*

Experiment No.	Protoporphyrin in blood	Protoporphyrin added	Concentration found	Concentration calculated	Recovery
	$\gamma$ per cent	$\gamma$	$\gamma$ per cent	$\gamma$ per cent	per cent
1	15.5	0.41	19.3	19.6	98.5
2	15.5	0.82	23.9	28.7	103.4
3	10.5	0.65	16.5	17.0	97.1
4	10.5	1.30	24.5	23.5	104.2
5	23.1	0.90	21.8	22.1	98.6

when pure protoporphyrin is added to 5 per cent HCl the recovery is practically quantitative. This is also true when pure protoporphyrin is added to the first HCl extract (as derived from blood samples). The data are shown in Table VII.

### *Comment*

The present investigation provides a relatively simple and accurate method for the quantitative estimation of the non-hemoglobin protoporphyrin in the erythrocytes. This method was needed in order that further studies might be made of the occurrence of this protoporphyrin under normal and pathological conditions. Reticulocytes were shown by Watson and Clarke (9) to be relatively rich in protoporphyrin. Seggel (6) believes that the protoporphyrin is contained in the "fluorescytes" and that these cells are not identical with reticulocytes. Obviously further studies of this problem are needed and these will require a method such as is herein described.



## SUMMARY

Methods are described for the quantitative determination of the non-hemoglobin protoporphyrin in the erythrocytes. The van den Bergh extraction procedure is employed with the exception that the time of shaking is much shortened, since it has been shown that prolonged shaking results in some loss. The amount of porphyrin in the final HCl extract may be determined either by measurement of absorption in the photoelectric colorimeter (No. 400 filter) or of fluorescence in the fluorophotometer. The excellent agreement obtained with these methods is regarded as proof of adequate purification of the final solution.

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# ENZYME STUDIES ON ISOLATED CELL NUCLEI OF RAT LIVER

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Although the isolation of nuclei of various types of cells dates back to the work of Miescher (1) who in 1868 isolated pus cell nuclei by a rather drastic method, little work has been published in more recent times on the preparation of nuclei by chemical methods sufficiently mild to leave the proteins and enzymes undenatured.

The work of Crossmon (2), Stoneburg (3), and Marshak (4, 5) has made it possible to obtain nuclei on a relatively large scale from normal rat liver cells or from rat tumor cells, in a pure state as far as separation from cytoplasm is concerned. Such preparations are very satisfactory for use in work on materials such as lipids or nucleic acid and acid-resistant nucleoproteins, but they are of no use for enzyme studies or for work with sensitive proteins, since the methods of preparation all involve such a low pH that in general enzymes and proteins must be denatured.

Since the work of Warburg (6), nuclei of bird erythrocytes have been available in relatively pure condition, but little if anything has been published on enzyme studies of these nuclei. Two new preparations of nuclei of bird erythrocytes are those of Laskowski (7) and Lan and Dounce.<sup>1</sup>

Zittle and Zitin (8) recently have isolated nuclei of mammalian spermatozoa and have investigated the cytochrome oxidase activity of the nuclei. Evidently nothing has been done on the enzymes of isolated nuclei of fish spermatozoa, which have been available for a long time (9, 10).

Nuclei of egg cells such as those of *Arbacia punctulata* can be prepared by a special centrifugation technique worked out by Harvey (11) and Harvey (12). Boell, Chambers, Glancy, and Stern (13) have investigated the cytochrome oxidase activity of the two half cells. The results of their work are included later in this paper.

Behrens and collaborators (14) have published a method for separation of nuclei from cereal germ cells, but in this case studies were made only on the types of nucleic acid present in cytoplasm and nucleus. Behrens has also been able to obtain nuclei from hepatic cells (15). The liver tissue is frozen and dried in the frozen state, followed by grinding and suspending in a carbon tetrachloride-benzene mixture. This suspension is centrifuged and the nuclei are concentrated in the bottom layer of the centrifuge tube.

<sup>1</sup> Lan, T. H., and Dounce, A. L., unpublished data.

Behrens reports that the nuclear concentrate obtained in this way contains nearly as high a concentration of arginase as does the upper cytoplasmic layer, while the concentration of lipase in the nuclei is small compared to that of the cytoplasm.

Application of the method of Behrens to the preparation of thymus cell nuclei by Mayer (16) does not seem to have yielded pure undamaged nuclei.

It should be noticed that by the method of Behrens nuclei are not prepared in undamaged condition, since the lipid must have been extracted to a considerable extent.

Lazarow (17) has reported the isolation of cell nuclei of guinea pig liver by forcing a finely ground liver suspension through bolting silk, but details are not given. The oxygen consumption of the nuclei, when various substrates were used, was studied.

Preparation of nuclei by micro dissection methods does not yield enough material for extensive chemical investigation.

In the following paragraphs there will be described a method for preparing cell nuclei of normal rat liver, which is believed to leave the enzymes and proteins undamaged. An account also will be given of the enzymes of these nuclei which so far have been investigated.

#### EXPERIMENTAL

*Preparation of Nuclei*—A mixture of roughly equal parts of cracked ice and distilled water<sup>2</sup> is made up to a volume of 500 cc., and 1.05 cc. of molar citric acid are added. This mixture is placed in a Waring blender or other suitable high speed mixer, and 100 gm. of frozen rat liver are added as rapidly as possible without stalling the blender. (The liver is frozen by placing it in the refrigerator unit, and it should be used as soon as it is frozen.) When all of the liver has been added to the mixer, it may be necessary to break up the mass of ground ice which accumulates at the top of the liquid and prevents proper mixing.

One now runs the mixer until all of the ice has melted. This should require about 10 to 15 minutes, depending upon the room temperature and the mixer employed. Then the finely divided material is strained by gravity through two layers of fine cheese-cloth containing about 20 threads per cm. As soon as the first piece of cheese-cloth is clogged, it is removed and a fresh piece is substituted. This is continued until all of the material has been strained. The cheese-cloth that has been used in this process is squeezed out and the liquid is collected in a separate beaker and is passed through fresh cheese-cloth into the already strained material. In this

<sup>2</sup> We have been unable to separate nuclei using physiological saline instead of distilled water. Mirsky and Pollister (18) have indicated that more than 60 per cent of the protein of a liver cell can be extracted by Ringer's solution without destroying the main outlines of cell structure.

way losses are cut to a minimum. The whole process of straining is then repeated with four layers of cheese-cloth instead of two. This time the straining proceeds without difficulty.

Next, the strained suspension is placed in 250 cc. centrifuge tubes and is centrifuged in an ordinary centrifuge for 20 minutes at a speed between 1500 and 2000 R.P.M. The speed is not critical. The nuclei, together with much cytoplasmic material, are thrown down. The supernatant is decanted to the point at which the first line of demarcation between supernatant and loosely packed sediment can be seen, and the supernatant is discarded. Care should be taken not to lose much of the loosely packed sediment. Now the sediment is stirred very thoroughly with enough distilled water to make a total volume of approximately 400 cc. Caprylic alcohol may be added to break foam. If necessary the material may be stirred in the blender for a few seconds to break up lumps, although this procedure introduces foam. Following this, the suspension is centrifuged in 250 cc. centrifuge tubes for 15 minutes. Much less loosely packed sediment will now be found in the bottom of the centrifuge tubes. The supernatant is discarded, leaving the packed and loosely packed sediment, which is stirred with about 400 cc. of distilled water and is centrifuged for 10 minutes in 250 cc. centrifuge tubes in an ordinary centrifuge. The sediment, which at this stage consists largely of nuclei, is washed with 200 cc. of distilled water, and is centrifuged at moderate speed (1000 to 1500 R.P.M.) for 5 minutes. The supernatant is discarded. The nuclei are now stirred with 200 cc. of distilled water, and are centrifuged at lower speed than before and for only 3 minutes. In this way most of the particles finer than nuclei are left in suspension. The washing with 200 cc. of distilled water and centrifuging for 3 minutes are now repeated twice. Before the last centrifugation it is of advantage to pass the suspension of nuclei through four layers of cheese-cloth.

Now the nuclei are well stirred with about 100 cc. of distilled water, and are allowed to stand in a 100 cc. cylinder for 45 minutes. The first 95 cc. of liquid, which contain most of the nuclei, are carefully decanted from the bottom 5 cc. which contain whole cells which have escaped being broken. This bottom layer is discarded. The nuclei are recovered from the first 95 cc. by centrifugation and are suspended in a small amount of distilled water. The final preparation is of a light reddish brown color. This color is caused by the presence of a small amount of adsorbed hemoglobin, the removal of which will be discussed later on.

*Discussion of Method*—The method depends largely upon the adjustment of pH to a value between 6.0 and 6.2 by means of M/475 citric acid. Much of the work of breaking the cell membranes is done by the blender. It is not known whether other acids can be substituted for citric acid.

Nuclei prepared by this method come from several types of cells, but

principally from hepatic cells, since these cells predominate in whole liver. A representative sample of the nuclei, photographed without staining, is shown in Fig. 1.

It is possible to break the cells at a pH of 6.5 or higher if one uses distilled water or very dilute sodium hexametaphosphate solution, but in such cases the nuclei themselves are rapidly disintegrated before they can be centrifuged off. This does not occur when purified nuclei are treated in this manner. On the other hand, if one employs pH values from 4.0 to 5.9, the cytoplasmic granules agglutinate so completely that they pack into a solid mass on centrifugation. Separation of nuclei from such a mass of agglutinated granules is practically impossible. If one uses a pH of

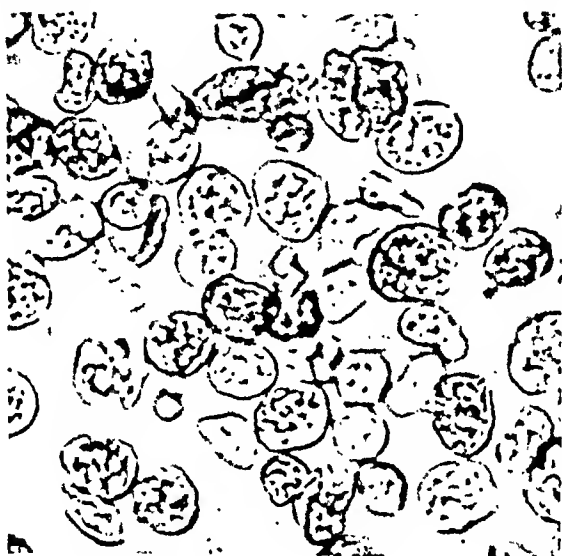


FIG. 1. Photograph of isolated cell nuclei of rat liver. Unstained preparation.  $\times 1170$ .

3.8 to 4.0, beautiful nuclei can be prepared, but this pH is so low that enzymes and proteins sensitive to acid must be partly or entirely destroyed, although the enzymes esterase and catalase do survive this treatment at least in part.

*Enzymes of Isolated Nuclei*—The main purpose of the following enzyme determinations has been to compare the activities per dry weight of enzymes in the isolated nuclei with the corresponding enzyme activities per dry weight of whole tissue. It should be kept in mind that even if the concentration of a particular enzyme in the nucleus is as great as its concentration in whole tissue, the total amount of enzyme present in the nucleus must be a relatively small fraction of the total amount present in

the cell, since the nucleus makes up only about 6 per cent of the total volume of a hepatic cell.

All of the following enzyme determinations, without exception, have been carried out at 25°.

*Arginase*—This enzyme was determined, somewhat roughly, as follows: To 1.0 cc. of 1 per cent arginine carbonate (*l* form), there was added 0.1 cc. of a suspension of nuclei or whole tissue. For nuclei the concentration of the suspension on a dry weight basis should be about 25 to 30 mg. per cc., and for whole liver about half this value. The mixture was incubated at 25° for a known length of time varying from 15 to 30 minutes. The reaction was stopped by adding 1.5 cc. of glacial acetic acid. Then 1.0 cc. of approximately 5 per cent xanthidrol in ethyl alcohol was added and the mixture was shaken and allowed to stand for at least 2 hours with intermittent shaking. The precipitated dioxanthylurea was centrifuged down, washed twice with 50 per cent acetic acid, twice with water, and then was transferred to a crucible, dried in the oven at 100°, and weighed. The arginase activity per dry weight of nuclei or whole tissue was measured by calculating the monomolecular reaction velocity constant  $K$  and dividing this by the dry weight of the tissue employed in the determination. The reaction as carried out above appears to be approximately monomolecular until half the substrate is used up. The pH of arginine carbonate is sufficiently high to make the addition of buffer unnecessary in a rough determination, as long as care is taken not to hydrolyze too much of the substrate.

By this procedure, the activity of arginase per dry weight of nuclei was found to vary from 40 to 50 per cent of the activity per dry weight of whole tissue. The value of  $K$  per gm. of dry weight averaged around 1.5 for nuclei and around 3.5 for whole liver. The addition of manganese salts to the nuclei did not increase their arginase activity appreciably.

*Catalase*—This enzyme was found to be almost lacking in activity in isolated nuclei, although the method of preparing the nuclei did not injure the catalase of the cytoplasm. Manometric technique was employed in the determination, since too small an amount of catalase is present in nuclei to be determined satisfactorily by the titration methods. 1.5 cc. of 0.01  $N$   $H_2O_2$  in  $M/150$  phosphate buffer of pH 6.8 were used as substrate, and to this was added in Warburg flasks 0.5 cc. of a suspension of nuclei or diluted whole liver suspension. The determination was carried out at 25°. The ratios of the initial slopes of the oxygen evolution curves were used in calculating the ratios of the activities. The results of a determination are shown in Fig. 2. The ratio of the activity of catalase per dry weight of whole liver suspension to its activity per dry weight in nuclei was found to be in the neighborhood of 2000:1.

It seems very unlikely that catalase, which according to its precipitability with ammonium sulfate and organic solvents must be more insoluble than arginase, should be washed out of the nuclei in the process of isolating them, while arginase should remain.

*Cytochrome Oxidase*<sup>3</sup>—The cytochrome oxidase activity of cell nuclei of fresh rat liver can be demonstrated qualitatively with *p*-phenylenediamine as substrate if one adds a little cytochrome *c* solution. Manometric measurements of the increase in oxygen consumption in the presence of added cytochrome *c*, with hydroquinone as substrate, showed that the nuclei possess cytochrome oxidase activity. This activity is greatly diminished or abolished by the addition of buffered HCN. For isolated nuclei, these manometric measurements indicate that the activity of cytochrome oxidase per dry weight of nuclei is at least 50 or 60 per cent of the activity of this enzyme per dry weight of whole liver suspension, when allowance is made for the fact that the cytochrome oxidase activity of isolated nuclei, kept

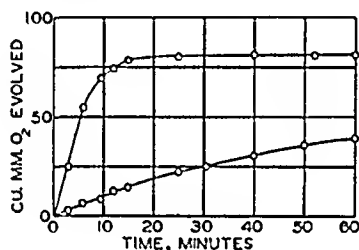


FIG. 2. Determination of catalase in isolated nuclei. Upper curve, 0.033 mg. of liver suspension, dry weight; lower curve, 6.2 mg. of nuclei, dry weight. 1.5 cc. of 0.01  $N$   $H_2O_2$  in  $M/150$  phosphate buffer, pH 6.8, plus 0.5 cc. of liver or nuclei suspension.

in the ice box at about  $3^\circ$ , falls off logarithmically at a rather high rate; so that for example at 24 hours about 24 per cent of the calculated initial activity has been lost. The decay of the enzyme activity of course may be higher than this during the time that the nuclei are being prepared.

In Fig. 3 the results of a typical determination of cytochrome oxidase in isolated nuclei are plotted graphically. The method of Brown and Goddard (19) was employed, with a pH of 6.8. The temperature was  $25^\circ$ . This gives somewhat lower values for oxygen consumption than are obtained at pH 7.4, but the base-line is so much lower that the determination is more satisfactory. Fig. 4 shows two curves for the rate of decay of cytochrome oxidase of two different preparations of isolated nuclei kept in the ice box at about  $3^\circ$ . As can be seen, the decay curve may vary somewhat from one preparation of nuclei to another.

<sup>3</sup> We wish to thank Professor D. R. Goddard of the Department of Botany for important advice and the gift of a considerable amount of cytochrome *c* which was used in carrying out experiments with cytochrome oxidase.

It might be added at this point that the  $QO_2$  for nuclei alone suspended in 0.05 M phosphate buffer at pH 6.8 is negligible. In isotonic phosphate buffer at pH 7.4 the  $QO_2$  is about 0.6 c.mm., and in distilled water about 1.0 c.mm.

In *Arbacia* eggs, Boell, Chambers, Glancy, and Stern (13) found a slightly different distribution of cytochrome oxidase than that reported above. They state that the increase in oxygen consumption upon the addition of *p*-phenylenediamine is somewhat greater for the lighter, nucleus-containing halves of the cell than for the heavier pigmented halves. Stern (20) has

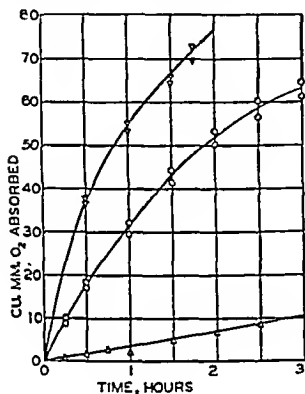


FIG. 3

FIG. 3. Cytochrome oxidase of whole liver and isolated nuclei. ▽, 10 mg. of whole liver suspension, dry weight, immediately after death of the animal; O, 11 mg. of isolated nuclei, dry weight, 7 hours after death of the animals; Δ, oxygen consumption of hydroquinone-cytochrome c without added liver suspension or nuclei. 1.0 cc. of 0.02 M hydroquinone in 0.1 M phosphate buffer, pH 6.8, plus 0.5 cc. of 0.0005 M cytochrome c, plus 0.5 cc. of liver suspension, nuclei, or water.

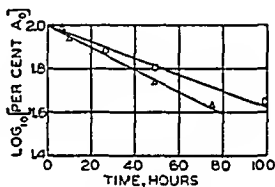


FIG. 4

FIG. 4. Decay of cytochrome oxidase in two different samples of isolated nuclei, about 3°.  $A_0$  = extrapolated initial activity at zero time.

reported that the cytochrome oxidase activity of heart muscle suspensions is associated with granules ranging from 50 to 196 mμ in diameter, which presumably come from cytoplasm.

The recent work of Zittle and Zitin, already mentioned (8), shows that the concentration of cytochrome oxidase in bull spermatozoa tails is about 24 times its concentration in the nuclei of the spermatozoa, and that the concentration of this enzyme in the mid-pieces is about 12 times its concentration in the nuclei.

**Esterase**—Semiquantitative determinations have been made by adding 0.1 cc. suspensions of whole tissue or nuclei to 5 cc. of water containing



0.1 cc. of molar ammonia-ammonium chloride buffer of pH 8.9, 0.2 cc. of methyl butyrate, and 2 drops of 0.04 per cent phenol red, and noting the length of time necessary for the indicator to become completely yellow at 25°. In this way the activity of esterase per dry weight of nuclei was found to average about 50 per cent of its activity per dry weight of whole liver suspension.

*Apoenzyme of Lactic Acid Dehydrogenase*<sup>4</sup>—This enzyme was present in nuclei, which showed an activity per dry weight of about 40 per cent of the corresponding activity of whole liver suspension. The Thunberg technique was employed with 2 per cent *dl*-sodium lactate as substrate, buffered to pH 7.0 with phosphate buffer, and with methylene blue as hydrogen acceptor. It was also necessary to add a small amount of coenzyme I from yeast, since without this the nuclei showed negligible activity. In determination of the activity of whole liver suspensions, excess coenzyme I also was added, since this was found to increase the activity of these suspensions materially. The coenzyme by itself had no measurable dehydrogenase activity.

*Alkaline Phosphatase*—This enzyme is the only one so far investigated which appears to be present in nuclei in higher concentration than in whole tissue. The substrate employed in the determinations was disodium phenyl phosphate in sodium barbiturate according to the method of King and Armstrong (21). The reaction as carried out was found to be of the zero order. The pH of the determination was about 9.45. The results were expressed in mg. of phenol liberated per mg. of tissue per hour. The activity per dry weight of alkaline phosphatase in isolated nuclei was found to average about 92 per cent higher than the activity of this enzyme per dry weight of whole liver suspension. Blanks were incubated for the same length of time as specimens to which enzyme was added.

*Acid Phosphatase*—Here the substrate employed again was disodium phenyl phosphate. The use of this substrate in determining serum phosphatase is discussed by Gutman and Gutman (22). After addition of the phenol reagent and filtration of the precipitated protein, the quantity of sodium carbonate added before the mixture was heated to develop color was adjusted so that the same color intensity per mg. of phenol was obtained in the acid phosphatase determinations as in the alkaline phosphatase determinations.

The activity per dry weight of acid phosphatase in nuclei was found to be only about 25 per cent of the activity per dry weight of this enzyme in whole liver suspension. The ratio of acid phosphatase activity to alkaline

<sup>4</sup> We are indebted to Professor J. B. Sumner of Cornell University for a sample of yeast codehydrogenase I which was used in determining the apoenzyme of lactic acid dehydrogenase.

phosphatase activity in the isolated nuclei was found to be about 1.5. This is much lower than the ratio of the activity of acid to alkaline phosphatase in whole rat liver. Greenstein *et al.* (23) reported the latter ratio to be about 6.2:1, presumably for Osborne-Mendel rats. We have found ratios as high as 13:1 for Wistar strain rats. We have not attempted to activate alkaline phosphatase in any manner in any of these determinations, but have analyzed the suspensions as soon as they were made. The addition of toluene-ethyl acetate mixture to the whole tissue suspension, which was ground in a homogenizer that completely disrupted most of the cells, did not result in any increase in activity of the enzyme.

One factor that might influence the ratio of the activity of acid to alkaline phosphatase in nuclei is the fact that although suspensions of whole nuclei were used in determining both alkaline and acid phosphatase, the high pH used in determining the alkaline phosphatase caused disruption of the nuclei, so that possibly more complete dispersion of the enzyme

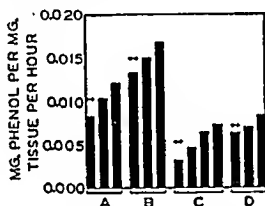


FIG. 5. Alkaline and acid phosphatase values for whole liver and isolated nuclei; dry weight basis. A = alkaline phosphatase in isolated nuclei; B = acid phosphatase in isolated nuclei; C = alkaline phosphatase in whole tissue; D = acid phosphatase in whole tissue divided by 10. Arrows indicate average values.

resulted than in the determination of acid phosphatase. In the latter determination the nuclei are not broken at the pH employed (5.0), and the reaction velocity may be influenced by the rate of diffusion of the substrate into the nuclei.

Fig. 5 shows the results of some of our phosphatase determinations expressed in mg. of phenol liberated per mg. of dry weight of tissue per hour, when 5 cc. of 1.1 per cent disodium phenyl phosphate, 5 cc. of buffer solution, and 0.1 cc. of enzyme suspension were incubated at 25°. For alkaline buffer, 2.3 per cent sodium barbital was used, and for acid buffer, 0.4 M acetate buffer of pH 5.0 was employed. It is of interest that Willmer (24) has reported that the chromosomes of dividing cells in tissue culture give a strongly positive phosphatase test.

*Succinic Acid Dehydrogenase*—This enzyme is generally found to be difficult to separate from cytochrome oxidase. Stern for instance (20) found that the activity of succinic acid dehydrogenase of heart muscle suspen-

sions was associated with particles ranging from 50 to 196  $m\mu$  in diameter, which also carried cytochrome oxidase activity.

With the Thunberg technique, as well as with the Warburg technique with added cytochrome *c*, the activity of succinic acid dehydrogenase of isolated cell nuclei of rat liver was found to be negligibly small or lacking entirely, although the enzyme could easily be demonstrated in the original supernatant solution after the nuclei were centrifuged down. This result is the opposite of the findings of Lazarow (17) who has reported that the increase in oxygen consumption of cell nuclei of guinea pig liver in the presence of succinic acid was greater than the corresponding increase for whole tissue.

*Preparation of Protein Extract from Nuclei*—Much of the protein of the nuclei will dissolve in 5 per cent sodium chloride solution at pH 6.0 to 6.3, leaving a residue which must be centrifuged down at about 15,000 R.P.M. in a centrifuge with a high speed attachment. This residue contains the bulk of the nuclear lipid, and still contains some nucleic acid. Protein also is present. The clear protein extract contains considerable nucleic acid and a small amount of hemoglobin which has been adsorbed by the nuclei. On standing in the ice box for some time, part of the protein of the extract precipitates. Protein also can be precipitated, together with nucleic acid, by dialysis or by acidification to pH 4.5 to 5.0. This protein extract has not yet been investigated to determine whether it contains all of the enzymes so far found in the nuclei, but it does contain the apoenzyme of lactic acid dehydrogenase and esterase and no doubt contains most of the others.

*Preparation of Nucleic Acid from Nuclei*—A sample of the protein extract described above was dialyzed for 18 hours against veronal buffer of pH 8.5. A precipitate which formed was centrifuged off. On treatment with 5 per cent sodium chloride solution part of this precipitate went into solution. The high speed centrifuge was then used to remove the insoluble residue of denatured protein, and the clear supernatant was added to 2 volumes of alcohol. A fibrous precipitate of crude sodium desoxyribonucleate which formed was centrifuged off and was dissolved in a small amount of saturated sodium chloride solution and was again centrifuged in the high speed centrifuge. The clear supernatant was again precipitated by alcohol. The precipitate was dissolved in a small amount of water and the alcohol precipitation was repeated twice. The sodium desoxyribonucleate thus obtained gave a highly viscous solution in water in a concentration of 0.3 to 0.4 per cent. This solution showed stream double refraction and gave a strong Dische reaction with diphenylamine. The absorption spectrum in the ultraviolet region was determined spectrographically by Dr. L. T. Steadman of the Department of Radiology. The absorption

spectrum, shown in Fig. 6, indicated that the material was relatively free from protein. With an approximate figure for the dry weight per cc. of the material obtained by drying 0.5 cc. of the solution, which had not been dialyzed, an absorption coefficient of about 1.8 was calculated, with the concentration expressed in gm. per cc. and the path length in cm.

The value of the absorption coefficient of a sample of thymus sodium desoxyribonucleate prepared in this laboratory according to Hammarsten was found to be 2.1. When the two solutions of sodium desoxyribonucleate were adjusted in concentration so that the intensity of the absorption at 2600 Å. was the same, the material prepared from the isolated nuclei gave a Dische reaction, measured in the photoelectric colorimeter, which was about 10 per cent higher than the intensity of the Dische reaction given by the thymus nucleic acid. More work would be necessary to determine the exact cause of this difference.

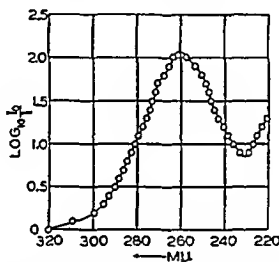


FIG. 6. Ultraviolet absorption spectrum of sodium desoxyribonucleate from isolated nuclei. Concentration, 0.0023 per cent approximately; path length, 5 cm.; absorption coefficient, 1.8 approximately, with concentration in gm. per cc. and path length in cm.

*Removal of Hemoglobin from Nuclei*—The nuclei as prepared by the method described in this paper are light reddish brown in color, owing to the presence of a small amount of adsorbed hemoglobin. This hemoglobin can be removed largely by one washing with Ringer's phosphate solution at pH 7.4, and entirely by two washings. However, Ringer's solution causes the nuclei to shrink and extracts relatively large amounts of protein even in the third washing. Only a trace of nucleic acid appears to be extracted. After three washings with Ringer's solution, the nuclei still show strong reactions for most of the enzymes investigated. Cytochrome oxidase appears to be increased slightly; acid and alkaline phosphatase, esterase, and arginase are relatively unchanged, and lactic acid dehydrogenase is still detectable but considerably diminished.

*Total Lipid of Nuclei*—The total lipid was determined by extracting samples of nuclei, dried by the lyophile process, in a small continuous ex-

tractor with a mixture of 3 parts of alcohol to 1 part of ether, and weighing the extracted lipid after evaporation of the solvent. Two determinations of total lipid on different samples of nuclei gave 10.7 and 10.8 per cent. Further extraction of the nuclei with chloroform-methanol mixture did not remove any more lipid. The lipid of the nuclei appears to be unstable, since it quickly develops an odor of rancidity.

### DISCUSSION

Table I gives a summary of the results of the investigation of enzymes of isolated cell nuclei of rat liver. From these results it seems clear that the nuclei contain several enzymes, at least, in relatively high concentra-

TABLE I  
*Enzymes of Isolated Cell Nuclei of Rat Liver*

Enzyme, coenzyme, etc.	Presence or absence in nucleus	Approximate enzyme concentration in nucleus expressed as per cent of enzyme concentration in whole tissue
Arginase.....	Present	40-50
Catalase.....	Extremely low	0.05
Cytochrome oxidase.....	Present	50-60 or higher
Esterase.....	"	50
Apoenzyme of lactic acid dehydrogenase..	"	40
Alkaline phosphatase.....	"	192
Acid phosphatase.....	"	25-30 or higher
Succinic acid dehydrogenase.....	Very low or absent	
Cytochrome c.....	Absent	
Coenzyme I.....	Extremely low or absent	
Riboflavin.....	Low or absent (?)	

tions. If one wishes to calculate roughly the percentage of the total amount of cellular enzyme that is present in the nucleus, data obtained by Marshak (5) for the ratio of the nuclear volume to the total cell volume can be used. For hepatic cells this was found by Marshak to be about 6:100.

The results of washing the nuclei with Ringer's solution at pH 7.4, which would be expected to elute adsorbed protein and which indeed does elute adsorbed hemoglobin but fails to remove the enzymes, show that it is very unlikely that the enzymes found in the nuclei are merely adsorbed from the cytoplasm. Moreover if the enzymes were adsorbed, it is improbable that the adsorption could be so specific as to exclude catalase, which is very easily adsorbed in the ordinary laboratory procedures. Finally, the concentration of most of the enzymes found to be present seems to be too high to make adsorption appear plausible.

It seems unlikely that much protein is lost from the nuclei in preparing them, since the last two or three washings, after being clarified in the high speed centrifuge, are almost protein-free. The pH of 6.0 to 6.2 used in the preparation is probably favorable for the retention of protein by the nuclei, especially in the presence of nucleic acid. On the other hand, Ringer's solution appears to extract protein from the nuclei every time it is used to wash them. It may require much more work to decide whether any protein is extracted during preparation of the nuclei.

In regard to certain constituents of relatively low molecular weight, it seems probable that they may be washed out in the preparation of the nuclei. For instance, coenzyme I is apparently absent, since it was necessary to add this material to obtain the activity of lactic acid dehydrogenase with isolated nuclei. We have found no evidence to indicate the presence of riboflavin in the nuclei. Cytochrome *c* appears to be absent, since it was necessary to add it in order to obtain cytochrome oxidase. Moreover, we have been unable to detect cytochrome *c* spectroscopically in isolated nuclei or extracts made from them, although we have tried many times. Cytochrome *c* is of such low molecular weight that it conceivably could have been washed out of the nuclei during their preparation.

All of the work reported in this paper refers to Wistar strain rats. We have, however, prepared nuclei from Osborne-Mendel strain rats and as far as we have made comparisons have found no marked differences in the activities of the enzymes.

In the future we hope to continue work on the enzymes of nuclei of normal rat liver and to extend the work to the nuclei of rat hepatomas.

We wish to acknowledge the financial support of The International Cancer Research Foundation of Philadelphia, Pennsylvania, which has made this work possible. Also we wish to thank Professor W. R. Bloor for his advice and encouragement throughout the course of this work.

#### SUMMARY

1. An improved method has been described for the preparation of nuclei of normal rat liver in such condition that their enzymes and proteins are believed to be undamaged.
2. The enzymes arginase, cytochrome oxidase, esterase, lactic acid dehydrogenase, alkaline phosphatase, and acid phosphatase have been found to occur in the nuclei in fairly high concentration. Catalase is extremely low. Succinic acid dehydrogenase is very low or absent. Cytochrome *c* and coenzyme I also are low or absent.
3. Reasons are given to show that the enzymes found to be present are in the nuclei and are not merely adsorbed.

4. The sodium salt of a highly polymerized desoxyribonucleic acid has been prepared from the nuclei.

5. The total lipid content of the nuclei was found to lie between 10.5 and 11.0 per cent.

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# ADENOSINE TRIPHOSPHATE IN MAGNESIUM ANESTHESIA\*

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Recent papers have given strong indication that myosin and adenosine triphosphatase may be identical (1-3) and have given renewed emphasis to the idea that adenosine triphosphate is the immediate source of energy for muscular work. One of the questions which immediately arises pertains to the form and function of adenosine triphosphatase in tissues which do not contain myosin, since there is abundant evidence that adenosine triphosphate may be the immediate source of energy for endergonic reactions in other tissues besides muscle (4). Work on this problem is dependent upon the availability of supplies of adenosine triphosphate. The present paper is an attempt to improve existing methods for the isolation of this compound, and, what is perhaps equally important, provides a possible explanation for the phenomenon of magnesium anesthesia (5).

Heilbrunn (6) has suggested that magnesium anesthesia might be due to a replacement of calcium by magnesium in "some clotting reaction essentially similar to the surface precipitation reaction," since it is well known that calcium will reverse the magnesium effect and restore consciousness to an animal anesthetized with magnesium. Although specific catalysts could not be identified at that time, Heilbrunn did show that isolated muscle fibers contracted progressively in  $\text{CaCl}_2$  solutions but failed to contract in  $\text{MgCl}_2$  solutions of the same strength. The recent work on myosin showed that calcium is a specific activator of the adenosine triphosphatase (myosin) system (3) and that magnesium inhibits this action (7). It was therefore logical to postulate that, in the animal anesthetized with magnesium, the muscles are unable to contract because calcium is blocked away from adenosine triphosphatase by the chemically similar magnesium ion and that a much higher yield of adenosine triphosphate should be obtained from the muscles of a magnesium-treated animal than from an untreated animal. The experiments presented below support this hypothesis, and the results show further that the same effects are obtained with brain tissue.

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† National Research Council Fellow in the Natural Sciences, 1942-43.



## EXPERIMENTAL

*Methods*

The effect of magnesium and calcium on the adenosine triphosphatase activity of tissues was determined by measuring the quantity of adenosine triphosphate (ATP) in the tissues after the special treatment. Both rats and rabbits were used, and the ATP was measured both by analysis and by actual isolation. In both cases, the procedure described by Needham (2) was used. For the isolation, this procedure was followed almost exactly, except that a Waring blender was used to disintegrate the tissue, and it was found necessary to increase the amount of water in Lohmann's reagent 3-fold to obtain solution. For the analytical experiments, the same procedure was followed as far as but not including precipitation as the mercuric salt. Tissue samples weighing approximately 1 gm. were removed from the animals and dropped into homogenizer tubes containing 10 per cent trichloroacetic acid. They were then rapidly disintegrated with a motor-driven stainless steel pestle. The ATP was then determined on the basis of the increase in inorganic phosphate after 7 minutes hydrolysis in normal HCl at 100°. In one experiment, the 7 minute phosphorus was measured directly on the trichloroacetic acid filtrate. Phosphorus was determined by the method of Fiske and Subbarow (8). In the case of the actual isolation of ATP, the material isolated was tested for purity on the basis of 7 minute phosphorus and on the basis of the ratio of total organic phosphorus to 7 minute phosphorus. For pure ATP, this ratio is 3.0:2.0.

*Analytical Studies*—Preliminary experiments were done to determine the amount of magnesium needed to produce anesthesia. A solution of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  was made up to contain 25 per cent  $\text{MgSO}_4$ . Various amounts were injected intraperitoneally, and 900 mg. per kilo were chosen as the proper initial dose. In some cases, this amount of magnesium failed to produce complete narcosis, and additional small amounts of magnesium sulfate were injected until cardiac failure resulted. The animals were then quickly decapitated; a sample of muscle and the entire brain were removed, weighed, and dropped into prepared homogenizer tubes. Parallel experiments were carried out with decapitated untreated animals and, as an additional control, with animals anesthetized with ether, since in the untreated animals the lowered ATP content might be attributed to the muscular contractions which occurred during decapitation. The results shown in Table I suggest that both muscle and brain from magnesium-treated animals contain more ATP than do the tissues from animals which have not been treated with magnesium. In Experiment 2, the ether anesthesia was maintained for exactly the same length of time as the magnesium anes-

thetia. In Experiment 4, the higher amount of ATP in every case is probably due to the omission of the extra manipulation involved in the precipitation of the barium salts. Bailey (3), on the basis of experiments with myosin, concluded that the calcium ion is a specific activator for adenosine triphosphatase. His observation led us to carry out the additional tests shown in Experiment 4, Table I. In addition to the determinations of ATP on the muscle samples from the various animals, an aliquot sample of muscle was minced and placed in a small volume of 0.05 per cent  $\text{CaCl}_2$ . A like sample of the muscle was placed in water. After 10 minutes incubation, the small pieces of muscle were removed from their respec-

TABLE I

*Adenosine Triphosphate Content of Rat Muscle and Brain in Magnesium Anesthesia*

Experiment No.	Treatment	ATP per gm. fresh tissue	
		Muscle	Brain
		<i>micromoles</i>	<i>micromole</i>
1	Decapitated	1.1	0.06
	Ether	1.3	0.09
	Magnesium, death in 40 min.	3.0	0.23
2	Decapitated	2.0	0.07
	Ether, anesthesia maintained 10 min.	3.1	0.06
	Magnesium, death in 10 min.	4.2	0.16
3	Decapitated	2.0	0.06
	Ether	1.0	0.11
	Magnesium, death in 10 min.	3.2	
	“ “ “ 40 “	4.9	0.25
4*	Decapitated, control	6.2	
	“ calcium-treated	3.0	
	Ether, control	4.3	
	“ calcium-treated	2.9	
	Magnesium, death in 30 min., control	8.5	
	“ “ “ 30 “ calcium-treated	5.4	

\* Analysis carried out directly on the trichloroacetic filtrate.

tive solutions and homogenized in trichloroacetic acid, and 7 minute phosphorus was determined on the filtrates. It is apparent that incubation in the calcium solution caused a decrease in the amount of ATP in every case, in accordance with Bailey's statement that calcium activates the breakdown of ATP by the muscle enzyme.

The above experiment (No. 4 in Table I) was confirmed and extended by an experiment with rabbits, on heart and diaphragm in addition to skeletal muscle. In this instance, the tissues were homogenized directly in magnesium sulfate or calcium chloride (0.1 per cent). This was done in the case of a magnesium-treated animal and a normal untreated animal which was

killed by a blow on the head. The results (Fig. 1) show, first, that the tissues vary considerably in their ATP content, skeletal muscle possessing most, diaphragm less, and heart muscle least; second, that the tissues from the magnesium-treated animal contain more ATP than do the corresponding tissues from the control animal; and third, that calcium in all cases decreases the ATP content of the untreated tissues in comparison with those homogenized in a solution containing magnesium.

*Isolation of Adenosine Triphosphate from Rabbit Muscle*—The analytical data indicate that skeletal muscle from magnesium-treated animals contains more ATP than muscle from untreated animals or from ether-anesthetized animals. Accordingly, larger scale experiments were carried out in which ATP was isolated from the skeletal muscle of rabbits. A total of eleven rabbits was killed, and ATP was isolated from their skeletal

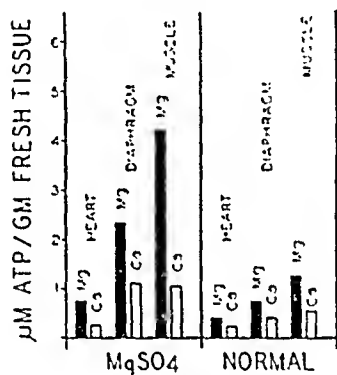


FIG. 1. Adenosine triphosphate content of tissues from a magnesium-treated and a normal rabbit as influenced by homogenization in calcium- and in magnesium-containing solutions.

muscles. The results are reported in Table II. Each sample was dried, weighed, and then analyzed for inorganic, total, and 7 minute phosphorus. From the data so obtained, the weight of barium adenosine triphosphate was calculated on the basis that it contained 4 moles of water per mole of ATP. (Lohmann and Schuster (9) reported 4 moles of water and pointed out that the amount of water depends upon the method of drying. Needham (2) reported 6 moles of water. Our purest preparation contained 99 per cent ATP if  $4\text{H}_2\text{O}$  and 103 per cent ATP if  $6\text{H}_2\text{O}$ ; the latter therefore was ruled out.) The ratio of total organic phosphorus to 7 minute phosphorus is also reported in Table II. The results are in harmony with the preliminary analytical data and support the conclusion that magnesium anesthesia may be due to the inability of the tissues to obtain energy by breaking down ATP and suggest that higher yields of ATP may be expected from magnesium-treated animals than from untreated animals.

The data in Fig. 1 were obtained from Rabbits 3A and 3B reported in Table II. The amount of ATP actually isolated from the magnesium-treated animal (Rabbit 3B) was 3.51 gm. per kilo of muscle. When the analytical data for this animal are calculated from the results in Fig. 1,

TABLE II  
*Isolation of Adenosine Triphosphate from Rabbit Muscle*

Rabbit No.	Treatment*	Total organic P 7 min. P	Ba <sub>2</sub> ATP·4H <sub>2</sub> O in product	Yield Ba <sub>2</sub> ATP·4H <sub>2</sub> O per kilo fresh muscle
			per cent	gm.
1A	Killed, untreated	3.5:2.0	96	1.55
1B	Injected with MgSO <sub>4</sub> , 700 mg. per kilo; died in 10 min.	3.1:2.0	81	2.10
1C	Injected with MgSO <sub>4</sub> , 500 mg. per kilo; died in 18 min.	2.9:2.0	97	2.70
2A	Killed, untreated	3.1:2.0	94	1.67
2B	Injected with MgSO <sub>4</sub> , 500 mg. per kilo; after 20 min., additional 125 mg. per kilo; after further 10 min., 125 mg. per kilo; after further 10 min., 175 mg. per kilo; death followed immediately	3.2:2.0	99	1.86
3A	Killed, untreated	3.2:2.0	89	2.56
3B	Injected with MgSO <sub>4</sub> , 500 mg. per kilo; after 20 min., additional 125 mg. per kilo; death in 5 min.	3.2:2.0	90	3.51
4A†	Killed with ether	3.0:2.0	83	1.85
4B†	Injected with MgSO <sub>4</sub> , 400 mg. per kilo; died in 12 min.	3.2:2.0	89	2.24
4C†	Injected with MgSO <sub>4</sub> , 200 mg. per kilo; after 30 min., additional 200 mg. per kilo; died in 15 min.	3.1:2.0	95	2.94
4D†	Injected with MgSO <sub>4</sub> , 250 mg. per kilo; after 30 min., additional 250 mg. per kilo; died in 20 min.	3.1:2.0	91	3.16
Average for controls .....				1.91
" " magnesium-treated .....				2.64

\* In the magnesium-treated animals, paralysis occurred soon after the first injection.

† Data on this group were kindly supplied by Dr. W. W. Umbreit and Mr. G. A. LePage.

the ATP content is calculated as 3.57 gm. per kilo of muscle. When similar calculations are made in the case of the untreated normal animal (Rabbit 3A), the amount actually isolated is 2.56 gm. per kilo as compared with only 1.54 gm. per kilo calculated from the analysis. The low figure in

the latter experiment is explainable on the basis of loss during homogenization of the sample in the magnesium solution before the magnesium actually reached the adenosine triphosphatase; in the case of the magnesium-treated animal, the magnesium had already reached the adenosine triphosphatase before the animal was killed, and homogenization in magnesium solution merely prevented loss of magnesium. In the tissues from which the ATP was actually isolated, the homogenization was carried out directly in trichloroacetic acid.

#### SUMMARY

1. Higher yields of adenosine triphosphate were obtained from the muscles of magnesium-anesthetized rabbits than from the muscles of untreated rabbits.

2. It was suggested that in magnesium anesthesia the magnesium ion may compete with the calcium ion for the surface of adenosine triphosphatase and thus prevent the breakdown of adenosine triphosphate.

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# DETERMINATION OF CALCIUM BY PRECIPITATION WITH PICROLONIC ACID AND POLAROGRAPHIC MEASUREMENT OF THE RESIDUAL PICROLONIC ACID

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The use of picrolonic acid for the determination of calcium has been described by several authors. All methods involve a separation of the precipitated calcium picrolonate,  $\text{Ca}(\text{C}_{10}\text{H}_7\text{O}_5\text{N}_3)_2 \cdot 2\text{H}_2\text{O}$ , from the supernatant liquid. The calcium picrolonate formed is subsequently determined either directly or indirectly. In the direct methods it is weighed or determined colorimetrically (1) or as carbon dioxide after combustion (2). In the indirect way the amount of picrolonic acid left in solution is determined volumetrically by titration with methylene blue (3). In a subsequent paper the latter method will be discussed in greater detail. In the direct methods a large excess of picrolonic acid (10-fold or more) is added and the precipitate is separated after 3 hours or a longer time of standing. The filtration or the centrifuging may involve difficulties when no well shaped crystals are formed, and also the washing may cause errors, since calcium picrolonate is appreciably soluble. In the method described in this paper the calcium is precipitated with a relatively slight excess of picrolonic acid, the excess of reagent being determined polarographically without filtering the precipitate. In this way errors due to incomplete separation by filtration or to solution of part of the precipitate are avoided. On the other hand complete precipitation of the calcium requires a relatively long time of standing (usually overnight), since only a slight excess of reagent is used in the precipitation.

## Reagents—

*Calcium chloride solution.* A 0.1004 M stock solution of calcium chloride was prepared by dissolving 14.7 gm. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  in water and diluting to 1 liter. The concentration of the solution was found by precipitating the calcium with sulfuric acid in 75 per cent alcohol and by weighing the calcium sulfate formed. The other calcium solutions used were prepared by diluting the stock solution.

*Picrolonic acid.* Saturated solutions which are about 0.01 M were prepared according to Dworzak and Reich-Rohrwig (4) by heating a sufficient amount of picrolonic acid recrystallized from 33 per cent acetic acid with water on a steam bath for 1 day and by filtering after cooling to room temperature. When the filtration was carried out after some days of standing, the solution remained clear for a long time and did not change its concentra-

tion as determined by titration with sodium hydroxide. The titration was carried out with 0.1 M carbonate-free sodium hydroxide which was added to the picrolonic acid solution from a micro burette with phenolphthalein as indicator. The end-point is sharp and the yellow color of picrolonate ions does not interfere. Two standard solutions were prepared. The first had an initial molarity of 0.01012, which had changed to 0.01035 M after 14 days. The second had a molarity of 0.0100 which remained the same after 2 months of standing. The solutions were kept in flasks of Pyrex glass. When kept in ordinary glass containers, some of the picrolonic acid precipitated on standing by reaction with calcium dissolved from the glass. The other solutions of picrolonic acid used were prepared by diluting the stock solutions.

TABLE I

*Characteristics of Capillary Used at 25° in 0.1 M Potassium Chloride at 41 Cm. of Mercury Pressure*

The values are expressed in volts.

	Potential of dropping mercury electrode against saturated calomel electrode					
	0	-0.3	-0.6	-0.9	-1.2	-1.5
Drop time, sec. ....	5.25	5.25	5.95	5.77	5.35	4.72
Mass, gm. per sec. ....	1.523	1.533	1.528	1.542	1.510	1.545
$m^{3/2}$ .....	1.745	1.75	1.79	1.79	1.75	1.73

### *Polarographic Behavior of Picrolonic Acid*

Since the determination of calcium was based on the polarographic measurement of the residual concentration of picrolonic acid, current-voltage curves of picrolonic acid were determined with the dropping mercury electrode under various conditions. The purpose was to find media in which the diffusion current was well defined and proportional to the concentration. The processes occurring in the reduction of picrolonic acid at the dropping mercury electrode will be discussed in connection with a study of the polarography of nitro compounds which is now being carried out in this laboratory. In the present paper only observations of interest for analytical applications of the waves of picrolonic acid are described. A pool of mercury in the electrolysis cell served as the anode. In 0.1 M potassium chloride at a mercury pressure of 41 cm. the characteristics of the capillary used were, at 25°, as shown in Table I.

In Fig. 1 current-voltage curves of picrolonic acid in potassium or lithium chloride solutions are shown. From these curves it is apparent that the presence of a maximum suppressor is necessary, and that camphor is espe-

cially suitable, while gelatin and thymol are less effective. In the presence of camphor (Curve 3) two distinct waves were obtained. The first one was horizontal at an applied E.M.F. between  $-1.0$  and  $-1.3$  volts, the second one at an applied E.M.F. more negative than  $-1.4$  volts. The solutions used in Fig. 1 were unbuffered. The appearance of the waves found in such solutions is greatly affected by the large change of pH close to the electrode during the reduction of the picrolonic acid.

Since calcium is precipitated from acid medium, a study was made of the current-voltage curves of picrolonic acid in acid solutions. In the presence of acids camphor was no longer the best maximum suppressor, gelatin being found much more efficient in this respect (see Figs. 2 and 3). In acetate buffers of pH 3.6 to 3.8 and in the presence of gelatin two waves were again

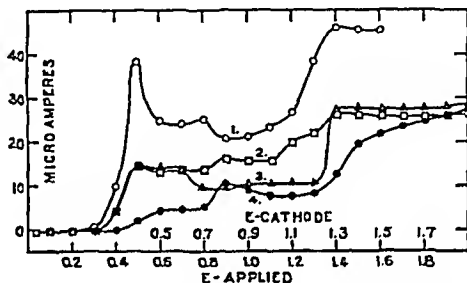


FIG. 1. Polarograms of picrolonic acid in unbuffered solutions. Curve 1,  $2 \times 10^{-3}$  M picrolonic acid, 0.1 M LiCl,  $20^\circ$ ; Curve 2,  $1.035 \times 10^{-3}$  M picrolonic acid, 0.1 M KCl, 0.036 per cent thymol,  $25^\circ$ ; Curve 3,  $1.035 \times 10^{-3}$  M picrolonic acid, 0.1 M KCl, 0.04 per cent camphor,  $25^\circ$ ; Curve 4,  $1.035 \times 10^{-3}$  M picrolonic acid, 0.1 M KCl, 0.1 per cent gelatin,  $25^\circ$ . The values are not corrected for residual current. Cathode potential refers to the saturated calomel electrode.

obtained. The first was not well defined, especially at higher concentrations of picrolonic acid (Fig. 3). On the other hand the second wave yielded a well defined diffusion current which remained practically constant at applied E.M.F. values of between  $-1.0$  and  $-1.2$  volts. The height of the second wave at  $E_a = -1.0$  volt was proportional to the concentration (Fig. 4). When the concentration of gelatin in the mixture was increased from about 0.02 to 0.1 per cent, the value of the diffusion current decreased by about 9 per cent.

In acetate buffers of pH 3.6 to 3.8 the presence of gelatin is not necessary for obtaining well defined diffusion currents. This is shown by the current-voltage curves in Fig. 5. The waves are similar to those obtained in the presence of gelatin, the first wave being badly defined, the second wave being well defined but showing a maximum. Under these conditions a



constant current is found at an applied E.M.F. of between  $-1.15$  and  $-1.3$  volts. The measurements reported in Table II and also the results of calcium determinations reported below show that the height of the wave at  $E_a = -1.2$  volts is proportional to the concentration of picrolonic acid in the absence of gelatin and is not affected by changes in the concentration of

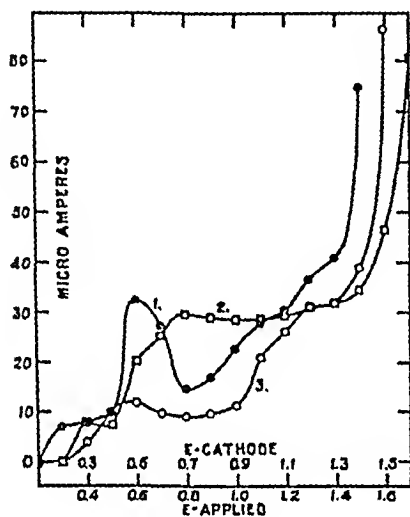


FIG. 2

FIG. 2. Polarograms of  $0.001035$  M picrolonic acid in acid medium at  $25^\circ$ . Curve 1,  $0.01$  M HCl,  $0.04$  per cent camphor; Curve 2,  $0.01$  M HCl,  $0.1$  per cent gelatin; Curve 3,  $0.8$  M HAc,  $0.1$  M  $\text{NH}_4\text{Ac}$ ,  $0.1$  M KCl,  $0.04$  per cent camphor. The values are not corrected for residual current. Cathode potential refers to the saturated calomel electrode.

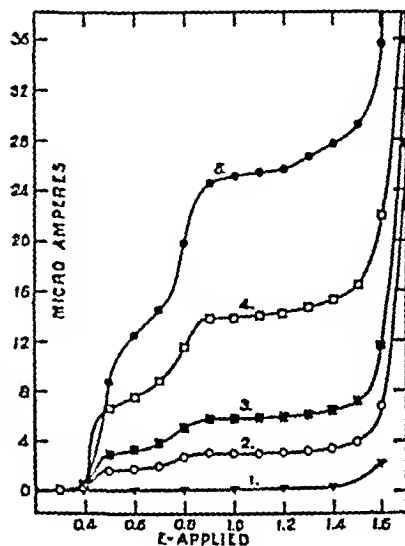


FIG. 3

FIG. 3. Polarograms of picrolonic acid in acetate buffer of pH 3.6 to 3.8 at  $25^\circ$ . Curve 1,  $0.08$  M HAc,  $0.01$  M  $\text{NH}_4\text{Ac}$ ,  $0.1$  M KCl,  $0.025$  per cent gelatin (residual current); Curve 2,  $1.049 \times 10^{-4}$  M picrolonic acid,  $0.079$  M HAc,  $0.0099$  M  $\text{NH}_4\text{Ac}$ ,  $0.099$  M KCl,  $0.0248$  per cent gelatin; Curve 3,  $2.07 \times 10^{-4}$  M picrolonic acid,  $0.08$  M HAc,  $0.01$  M  $\text{NH}_4\text{Ac}$ ,  $0.1$  M KCl,  $0.02$  per cent gelatin; Curve 4,  $5.062 \times 10^{-4}$  M picrolonic acid,  $0.076$  M HAc,  $0.0095$  M  $\text{NH}_4\text{Ac}$ ,  $0.095$  M KCl,  $0.0238$  per cent gelatin; Curve 5,  $9.121 \times 10^{-4}$  M picrolonic acid,  $0.073$  M HAc,  $0.0091$  M  $\text{NH}_4\text{Ac}$ ,  $0.091$  M KCl,  $0.0228$  per cent gelatin. The values are not corrected for residual current. Cathode potential (versus saturated calomel electrode) =  $(E_{\text{applied}} - 0.1 \text{ volt})$ .

acetate buffer or of indifferent electrolyte. Therefore, no gelatin was used in all later experiments.

### *Solubility of Calcium Picrolonate*

The precipitation of calcium was carried out in an acetate buffer and in the presence of alkali chloride which served to define the anode potential

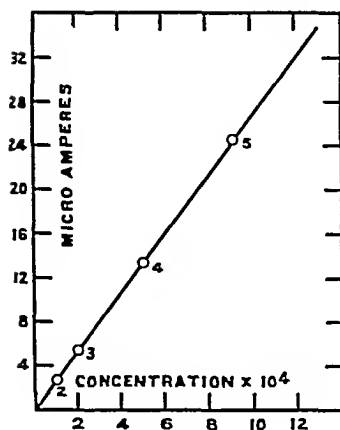


FIG. 4

FIG. 4. Diffusion currents of increasing picrolonic acid concentrations. The number of the points refers to the corresponding curves in Fig. 3. Correction applied for residual current.  $E_{\text{applied}} = -1.0$  volt,  $25^\circ$ .

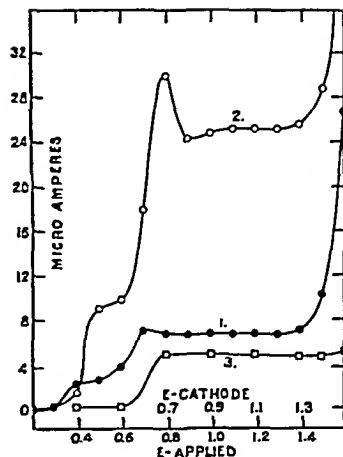


FIG. 5

FIG. 5. Polarograms of picrolonic acid in acetate buffer of pH 3.6 to 3.8 without gelatin. Curve 1,  $2.70 \times 10^{-4}$  M picrolonic acid, 0.08 M HAc, 0.01 M  $\text{NH}_4\text{Ac}$ , 0.1 M KCl; Curve 2,  $1.035 \times 10^{-3}$  M picrolonic acid, 0.08 M HAc, 0.01 M  $\text{NH}_4\text{Ac}$ , 0.1 M KCl; Curve 3, (for comparison)  $1.035 \times 10^{-3}$  M  $\text{CdCl}_2$ , 0.1 M HAc, 0.0125 M LiAc, 0.1 M LiCl. The values are not corrected for residual current. Cathode potential refers to the saturated calomel electrode.

TABLE II

Relation between Diffusion Current of Picrolonic Acid in Acetate Buffer and Concentration at  $E_a = -1.2$  Volts

Buffer A, 0.1 M HAc, 0.0125 M LiAc, 0.1 M LiCl; Buffer B, 0.05 M HAc, 0.0063 M LiAc, 0.05 M LiCl.

Buffer	0.01035 M picrolonic acid added to 10 ml. buffer	Concentration of picrolonic acid	Diffusion current corrected for residual current	Microamperes Concentration
	ml.	$\mu \times 10^3$	microamperes	$\times 10^3$
A	0.991	0.933	21.4	22.9
"	1.490	1.342	30.2	22.5
"	1.890	1.648	36.9	22.4
"	2.482	2.06	45.7	22.2
"	3.988	2.951	63.4	21.5
B	2.045	1.77	39.8	22.5

in the subsequent polarographic determination. Most alkali picrolonates are not very soluble. A  $6.72 \times 10^{-3}$  M picrolonic acid solution which

was 0.1 M in alkali chloride or in magnesium chloride yielded, after standing overnight in the ice box or in a cool room (at about 10°) considerable precipitates with  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Mg}^{++}$ , while the solution remained clear with lithium chloride. When the solution was only 0.0625 M in sodium chloride instead of 0.1 M, it also remained clear. Since calcium picrolonate crystallizes slowly, considerable errors may occur when the concentration of alkali ions is too large. Lithium picrolonate being the most soluble of the alkali salts, the following buffer mixture was used: 1 M lithium chloride, 1 M acetic acid, 0.125 M lithium acetate (pH 3.6 to 3.8). This mixture, abbreviated in the following as "buffer," was diluted about 10 times in the actual reaction medium.

For the determination of the solubility of calcium picrolonate pure crystals of this salt were added to the solutions given in Table III. After standing overnight at 20° and occasional shaking the concentrations of

TABLE III

*Solubility of Calcium Picrolonate in Excess of Picrolonic Acid at 20°*

All mixtures contained 10 ml. of buffer in 100 ml.

Molarity of picrolonic acid used	Final concentration of picrolonic acid found	Solubility of Ca picrolonate in M $\text{Ca}^{++}$	Solubility product
	M	$\times 10^4$	$\times 10^{11}$
$2.59 \times 10^{-4}$	$4.82 \times 10^{-4}$	2.41	5.6
$5.175 \times 10^{-4}$	$5.43 \times 10^{-4}$	1.42	4.2
$1.035 \times 10^{-3}$	$6.56 \times 10^{-4}$	0.693	3.0
$1.55 \times 10^{-3}$	$1.085 \times 10^{-3}$	0.025	
$1.55 \times 10^{-3}$	$1.55 \times 10^{-3}$	0	
$2.59 \times 10^{-3}$	$2.54 \times 10^{-3}$	0	

picrolonic acid were determined polarographically at 20° without filtration. The solubility of calcium picrolonate is appreciable. The value found in the buffer alone was  $2.41 \times 10^{-4}$  M  $\text{Ca}^{++}$ , or 9.6 mg. of Ca per liter. This is in agreement with the value of 9 mg. of  $\text{Ca}^{++}$  per liter at 21° in water given by Dworzak and Reich-Rohrwig (4). The solubility product was calculated with the assumption that the picrolonic acid is completely ionized in the buffer used. From titration curves of picrolonic acid with sodium hydroxide with the glass electrode it appears that this assumption is justified. It is seen that the solubility product ( $\text{Ca}^{++}$ ) (picrolonate ion)<sup>2</sup> is of the order of  $5 \times 10^{-11}$  at 20°. No calculations of the product have been made from experiments in which the concentration of picrolonic acid was greater than 0.001 M. The solubility of the calcium picrolonate then becomes so small that the experimental error in its indirect determination is too great to permit a reliable value of the solubility product to be calcu-

lated. From the analytical view-point it is significant that the solubility of calcium pierolonate becomes negligibly small when the excess of pierolonic acid is equal to or larger than  $1.5 \times 10^{-3}$  M. Fig. 6 (Curve 2) shows the solubility of calcium pierolonate in a given excess of pierolonic acid in the buffer solution diluted 10 times. In the actual calcium determinations the excess of pierolonic acid used was of the order of 1 to  $2 \times 10^{-3}$  M. The solubility of calcium pierolonate in  $1 \times 10^{-3}$  M pierolonic acid is  $2.5 \times 10^{-5}$  M Ca. Thus when in a calcium determination a final concentration of pierolonic acid of  $1.05 \times 10^{-3}$  M was found, the concentration corresponding to the amount of unchanged pierolonic acid in the solution was  $(1.05 - 0.05) \times 10^{-3}$  M or  $1.00 \times 10^{-3}$  M.

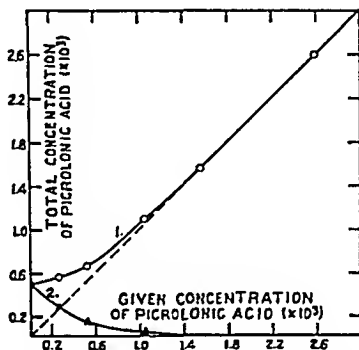


FIG. 6. Solubility of calcium pierolonate in 0.1 M HAc, 0.0125 M LiAc, 0.1 M LiCl at 20°. Curve 1, total concentration of pierolonic acid (sum of given concentration of pierolonic acid plus dissolved calcium pierolonate); Curve 2, concentration of calcium pierolonate at a given concentration of pierolonic acid.

#### *Determination of Calcium in Absence of Interfering Substances*

Since the crystallization of calcium pierolonate is slow and the solubility appreciable, an amperometric titration was not feasible. The determination must be carried out by adding an excess of pierolonic acid and measuring the amount left in solution. In order to add the appropriate amount of pierolonic acid to an unknown solution the calcium content of the solution should be known approximately. The approximate value of the calcium concentration can be obtained by noticing the time required for the first appearance of a precipitate of calcium pierolonate when the solution is mixed with buffer and pierolonic acid. Some results of such experiments at 20° are given in Table IV. The time for the appearance of the precipitate is increased more or less by the presence of other substances. Hence the test must be carried out with unknowns and standards of corresponding

composition. The approximate determination of the calcium content can also be carried out by adding oxalate solution to the reaction mixture and by comparing the turbidity with that obtained with standards. It was found that the turbidity was not affected by the presence of 0.25 M potassium chloride in calcium solutions over a concentration range of from 1.5 to  $10 \times 10^{-3}$  M. When the calcium solution was  $10^{-3}$  M, the turbidity was considerably weaker in the presence of 0.25 M potassium chloride than in the absence of potassium chloride.

*Determination of Calcium*—Table V contains the results of determinations of calcium in solutions, the concentration of which varied between  $10^{-3}$  and  $10^{-2}$  M. After the solutions had stood overnight in a cool room (about  $10^\circ$ ), the amount of picrolonic acid left was determined at  $20^\circ$  polarographically ( $E_a = -1.2$  volts) either by using the method of an "internal standard" or by running calibrations between determinations. These procedures were used in order to take care of any fluctuations of the capillary

TABLE IV

*Time of Appearance of Precipitate of Calcium Picrolonate*

To 1 ml. of calcium solution, 0.3 ml. of buffer and 2 ml. of 0.01 M picrolonic acid were added ( $20^\circ$ ).

Molarity of Ca solution	Time for appearance of ppt.
0.01	20-25 sec.
0.005	About 1 min.
0.003	" 1.5 min.
0.002	" 2 min.
0.001	2-3 min.

constant over longer periods of time (5). The first eleven experiments show that calcium in a concentration range between 0.01 and 0.001 M can be determined with an accuracy of 1 or 2 per cent. Experiments 12 to 15 illustrate the influence of the temperature at which the samples are kept overnight. When the temperature was about  $15^\circ$ , the precipitation from 0.001 M calcium solution was not complete. When, on the other hand, the 0.01 M calcium solution was placed overnight in the ice box, the results were consistently high. When the concentration of calcium was  $5 \times 10^{-3}$  M or less, correct results were found after standing overnight in the ice box. From the practical point of view it is most convenient to keep the mixtures overnight in an ice box. Under these conditions good results are obtained with concentrations of from 1 to  $5 \times 10^{-3}$  M calcium. When the concentration is larger, we recommend that the mixtures stand overnight at a temperature of  $10$ – $20^\circ$ .

The precipitation requires considerable time, dependent on the calcium concentration and on the presence of other salts. Even from a pure 0.01 M

TABLE V  
Determination of Calcium As Picronate by Polarographic Measurement of Excess of Picronic Acid

Experiment No.	Ca added	Buffer added	Picronic acid added	Picronic acid left (concentrations corrected for solubility if necessary)	Ca picronate pptd., in terms of picronic acid		Error
					Calculated	Found	
		ml.			micromoles	micromoles	per cent
1	5 ml. $1.004 \times 10^{-2}$ M	2	12.5 ml. $0.01$ M = 125*	$1.20 \times 10^{-3}$ M = 24.0*	100.4	100.4	0
2	5 " $1.004 \times 10^{-2}$ "	2	12.5 " $0.01035$ M = 129.5	$1.405 \times 10^{-3}$ " = 27.4	100.4	102.1	+1.7
3	5 " $1.004 \times 10^{-2}$ "	2	12.5 " $0.01035$ " = 129.5	$1.545 \times 10^{-3}$ " = 30.1	100.4	99.4	-1.0
4	5 " $1.004 \times 10^{-2}$ "	2	15 ml. $0.01$ M = 150	$2.15 \times 10^{-3}$ " = 47.3	100.4	102.7	+2.3
5	5 " $5.02 \times 10^{-3}$ "	1	7 " $0.01035$ M = 72.5	$1.75 \times 10^{-3}$ " = 22.8	50.2	50.7	+1.0
6	5 " $5.02 \times 10^{-3}$ "	1	6.5 ml. $0.01035$ M = 67.2	$1.26 \times 10^{-3}$ " = 15.8	50.2	51.4	+2.4
7	5 " $2.51 \times 10^{-3}$ "	1	4 ml. $0.01035$ M = 41.4	$1.60 \times 10^{-3}$ " = 16.0	25.1	24.8	-1.2
8	5 " $2.51 \times 10^{-3}$ "	0.5	3.5 ml. $0.01035$ M = 36.2	$1.23 \times 10^{-3}$ " = 11.1	25.1	25.1	0
9	10 " $1.004 \times 10^{-2}$ "	1	3.5 " $0.01035$ " = 36.2	$1.095 \times 10^{-3}$ " = 15.9	20.03	20.3	+1.1
10	10 " $1.004 \times 10^{-2}$ "	1	3.5 " $0.01$ M = 35.0	$1.00 \times 10^{-3}$ " = 14.5	20.03	20.5	+2.1
11	10 " $1.004 \times 10^{-2}$ "	1	3.5 " $0.01035$ M = 36.2	$1.135 \times 10^{-3}$ " = 16.46	20.03	19.74	-1.7
12	10 " $1.004 \times 10^{-2}$ "†	1	3.5 " $0.01$ M = 35.0	$1.365 \times 10^{-3}$ " = 19.7	20.03	15.3	-29
13	10 " $1.004 \times 10^{-2}$ "†	1	4.5 " $0.01$ " = 45.0	$1.865 \times 10^{-3}$ " = 28.9	20.03	16.1	-20
14	5 " $1.004 \times 10^{-2}$ "†	2	12.5 " $0.01$ " = 125.0	$1.08 \times 10^{-3}$ " = 21.2	100.4	103.8	+3.4
15	5 " $1.004 \times 10^{-2}$ "†	2	12.5 " $0.01$ " = 125.0	$1.06 \times 10^{-3}$ " = 20.7	100.4	104.3	+3.9

\* In micromoles.

† Kept overnight at about 15°.

‡ Kept overnight in ice box.

calcium solution the crystallization is still incomplete after 4 hours of standing at 20°. For this reason it is recommended that the mixtures always stand overnight.

The determinations of calcium were found to be well reproducible when the picrolonic acid used had been recrystallized from 33 per cent acetic acid. When the picrolonic acid, in addition, also had been recrystallized from 95 per cent ethanol, a disturbing effect was observed. The crystals of the precipitated calcium picrolonate were not so well developed as the crystals obtained with picrolonic acid recrystallized from acetic acid only. With the solution of picrolonic acid recrystallized from alcohol correct results were obtained in the determination of 0.001 and 0.01 M calcium solutions. In the determination of 0.0025 M calcium solutions, however, the calcium content was always found 7 to 8 per cent low, corresponding to an apparently abnormally large diffusion current of the residual picrolonic acid. At this calcium concentration part of the precipitate was in a slimy form. The error was eliminated by filtering the suspension of calcium picrolonate

TABLE VI  
*Estimate of Calcium Content in Unknown Solutions*

Sample No.	Time for appearance of ppt.	Estimated concentration of Ca
	<i>min.</i>	$M \times 10^3$
1	About 2.2	About 2
2	" 1	" 5
3	" 1.5-2	" 3

through a sintered glass crucible before the polarographic determination. Hence, it appears that under the working conditions the suspended calcium picrolonate contributed to the measured diffusion current. It is, therefore, recommended to recrystallize the picrolonic acid only from 33 per cent acetic acid.

*Determination of Calcium in Unknown Solutions*—The results of the determinations of three unknown solutions are reported in order to illustrate that the procedure yields reliable results. The preliminary test carried out with 1 ml. of solution, 0.3 ml. of buffer, and 2 ml. of 0.01 M picrolonic acid gave the results shown in Table VI. On the basis of these figures determinations were made in which the mixtures were kept in an ice box overnight (Table VII). The molarities were, Sample 1,  $2.71 \times 10^{-3}$  M; Sample 2,  $5.02 \times 10^{-3}$  M; Sample 3,  $3.36 \times 10^{-3}$  M. The results show that for most practical purposes one determination is sufficient.

*Determination of Calcium in Presence of Na, K, NH<sub>4</sub>, Mg, SO<sub>4</sub>, and PO<sub>4</sub>*

Alkali metals and magnesium interfere when present in large concentrations because of the limited solubility of their picrolonates. Sulfate and

phosphate might interfere by coprecipitation of their calcium compounds. An interference by magnesium is especially to be expected. According to Dworzak and Reich-Rohrwig (4) the solubility of  $\text{Mg}(\text{C}_{10}\text{H}_7\text{O}_5\text{N}_3)_2 \cdot 4\text{H}_2\text{O}$

TABLE VII  
*Determination of Calcium in Unknown Solutions*

Reaction Mixture	Sample 1	Sample 2	Sample 3
A	10 ml. unknown, 2 ml. buffer, 10 ml. 0.01 M picrolonic acid (allows determination up to $3.75 \times 10^{-3}$ M Ca)	5 ml. unknown, 1 ml. buffer, 7.5 ml. 0.01 M picrolonic acid (allows determination up to $6.5 \times 10^{-3}$ M Ca)	10 ml. unknown, 2 ml. buffer, 9 ml. 0.01 M picrolonic acid (allows determination up to $3.5 \times 10^{-3}$ M Ca)
B	10 ml. unknown, 2 ml. buffer, 7.5 ml. 0.01 M picrolonic acid (allows determination up to $2.75 \times 10^{-3}$ M Ca)	5 ml. unknown, 2 ml. buffer, 9 ml. 0.01 M picrolonic acid (allows determination up to $8 \times 10^{-3}$ M Ca)	5 ml. unknown, 1 ml. buffer, 6 ml. 0.01 M picrolonic acid (allows determination up to $5 \times 10^{-3}$ M Ca)
C		5 ml. unknown, 2 ml. buffer, 10 ml. 0.01 M picrolonic acid (allows determination up to $9 \times 10^{-3}$ M Ca)	
Amounts of Ca found in 10 ml. solution			
	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
A	27.15	51.2	32.7
B	26.88	51.3	33.8
C		49.7	
Correct value...	27.11	50.20	33.63
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A. Error	+0.15	+2.0	-2.8
B. "	-0.85	+2.2	+0.5
C. "		-1.0	
Average error....	-0.35	+1.1	-1.15

is 7.9 mg. of Mg per liter at 20°, only 1.45 times greater than the molar solubility of calcium picrolonate. According to these authors, magnesium forms stable supersaturated solutions, so that calcium can be determined in the presence of the 10-fold amount of magnesium (molar ratio 16.5:1). We



TABLE VIII—Determination of Calcium in Presence

Ex- peri- ment No.	Ca solution			Buffer added	Picrolonic acid added
	ml.	Molarity in Ca	Molarity of foreign salts		
1	5	$1.004 \times 10^{-2}$	0.1 NaCl	2	12.65 ml. 0.01035 M = 131.0
2	5	$1.004 \times 10^{-2}$	0.25 "	2	12.5 " 0.01035 M = 129.5
3	10	$2.505 \times 10^{-3}$	0.25 "	2	8 ml. 0.01035 M = 82.8
4	5	$2.51 \times 10^{-3}$	0.25 "	1	5 " 0.01 M = 50.0
5	10	$1.001 \times 10^{-3}$	0.25 "	1	3.5 ml. 0.01 M = 35.0
6	10	$1.002 \times 10^{-3}$	0.1 "	1	4.5 " 0.01 " = 45.0
7	5	$1.004 \times 10^{-2}$	0.25 KCl	2	12.5 " 0.01035 M = 129.5
8	5	$1.001 \times 10^{-2}$	0.1 "	2	12.5 " 0.01035 " = 129.5
9	10	$2.51 \times 10^{-3}$	0.25 "	2	8 ml. 0.01 M = 80.0
10	10	$1.004 \times 10^{-3}$	0.25 "	1	3.5 ml. 0.01035 M = 36.2
11	5	$1.004 \times 10^{-2}$	0.25 $\text{NH}_4\text{Cl}$	2	13 ml. 0.01 M = 130.0
12	10	$1.004 \times 10^{-3}$	0.1 "	1	4 " 0.01 " = 40.0
13	10	$1.004 \times 10^{-3}$	0.25 "	1	4 " 0.01 " = 40.0
14	5	$1.004 \times 10^{-2}$	0.1 $\text{MgCl}_2$	2	12.5 ml. 0.01035 M = 129.5
15	5	$1.004 \times 10^{-2}$	0.25 "	2	12.5 " 0.01035 " = 129.5
16	10	$2.51 \times 10^{-3}$	0.25 "	2	8 ml. 0.01035 M = 82.8
17	10	$1.004 \times 10^{-3}$	0.25 "	1	3.5 ml. 0.01035 M = 36.2
18	5	$1.004 \times 10^{-2}$	0.25 KCl	2	12.5 " 0.01 M = 125.0
19	5	$1.004 \times 10^{-2}$	0.25 $\text{MgCl}_2$	2	12.5 " 0.01 " = 125.0
20	5	$1.004 \times 10^{-2}$	0.15 $\text{Li}_2\text{SO}_4$	2	12.5 " 0.01 " = 125.0
21	5	$1.004 \times 10^{-2}$	0.25 $\text{NaH}_2\text{PO}_4$	2	12.5 " 0.01 " = 125.0
22	5	$1.004 \times 10^{-2}$	0.25 "	2	12.5 " 0.01 " = 125.0
23	5	$1.004 \times 10^{-2}$	0.15 $\text{Li}_2\text{SO}_4$	2	12.5 " 0.01 " = 125.0
24	5	$1.004 \times 10^{-2}$	0.25 $\text{NaH}_2\text{PO}_4$	2	15 ml. 0.01 M = 150.0
25	10	$1.004 \times 10^{-3}$	0.25 "	1	3.5 ml. 0.01 M = 35.0
26	10	$1.004 \times 10^{-3}$	0.25 "	1	3.5 " 0.01 " = 35.0
27	10	$1.004 \times 10^{-3}$	0.25 "	1	4.5 " 0.01 " = 45.0
28	10	$1.004 \times 10^{-3}$	0.15 $\text{Li}_2\text{SO}_4$	1	3.5 " 0.01 " = 35.0
29	10	$1.004 \times 10^{-3}$	0.15 "	1	3.5 " 0.01 " = 35.0
30	5	$1.004 \times 10^{-2}$	0.25 "	2	12.5 " 0.01 " = 125.0
31	5	$1.004 \times 10^{-2}$	0.25 "	2	12.5 " 0.01 " = 125.0
32	10	$1.004 \times 10^{-3}$	0.25 "	1	3.5 " 0.01 " = 35.0

\* In micromoles.

*Other Salts after Standing Overnight at About 10°*

Picrolonic acid left (concentrations corrected for solubility if necessary)	Ca picrolonate formed, in terms of picrolonic acid		Error	Remarks
	Calculated	Found		
	micromoles	micromoles	per cent	
$1.48 \times 10^{-3} M = 29.0^*$	100.4	102.0	+1.6	
$1.52 \times 10^{-3} " = 29.65$	100.4	99.85	-0.55	
$1.66 \times 10^{-3} " = 33.2$	50.1	49.6	-1.0	
$2.26 \times 10^{-3} " = 24.8$	25.1	25.2	+0.4	
$1.32 \times 10^{-3} " = 19.1$	20.08	15.9	-21	Kept overnight only at about 15°
$1.61 \times 10^{-3} " = 24.9$	20.04	20.1	+0.3	Kept overnight in ice box
$1.61 \times 10^{-3} " = 31.4$	100.4	98.1	-2.3	
$1.52 \times 10^{-3} " = 29.65$	100.4	99.85	-0.55	
$0.9 \times 10^{-3} " = 18.0$	50.2	62.0	+23.5	Pptn. of K picrolonate
$0.35 \times 10^{-3} " =$	20.08			Practically all picrolonic acid used up by K pptn.
$1.475 \times 10^{-3} " = 29.5$	100.4	100.5	+0.1	Kept overnight at 20°
$1.26 \times 10^{-3} " = 18.9$	20.08	21.1	+5.1	" " in ice box
$0.42 \times 10^{-3} " =$	20.08			" " " " "
				{ Pptn. of NH <sub>4</sub> picrolonate
$1.46 \times 10^{-3} " = 28.4$	100.4	101.1	+0.7	
$1.445 \times 10^{-3} " = 28.2$	100.4	101.3	+0.9	
$1.62 \times 10^{-3} " = 32.4$	50.2	50.4	+0.4	
$1.085 \times 10^{-3} " = 15.75$	20.08	20.45	+1.8	
$1.562 \times 10^{-3} " = 30.5$	100.4	94.5	-5.9	7 hrs. in ice only. Pptn. still incomplete
$1.275 \times 10^{-3} " = 24.85$	100.4	100.15	-0.25	6 hrs. in ice only. Pptn. complete
$1.21 \times 10^{-3} " = 23.6$	100.4	101.4	+1.0	
$1.185 \times 10^{-3} " = 23.1$	100.4	101.9	+1.5	
$1.10 \times 10^{-3} " = 20.7$	100.4	104.3	+3.9	Kept overnight in ice box
$1.09 \times 10^{-3} " = 20.5$	100.4	104.5	+4.1	" " " " "
$2.19 \times 10^{-3} " = 48.2$	100.4	101.8	+1.4	" " " " "
$0.99 \times 10^{-3} " = 14.35$	20.08	20.65	+2.8	" " " " "
$1.05 \times 10^{-3} " = 15.2$	20.08	19.8	-1.4	" " " " "
$1.17 \times 10^{-3} " = 18.1$	20.08	26.9	+34	" " " " "
				{ Pptn. of Na picrolonate
$0.99 \times 10^{-3} " = 14.35$	20.08	20.65	+2.8	Kept overnight in ice box
$0.99 \times 10^{-3} " = 14.35$	20.08	20.65	+2.8	" " " " "
$1.63 \times 10^{-3} " = 31.8$	100.4	93.2	-7.2	" " " " "
$1.60 \times 10^{-3} " = 31.2$	100.4	93.8	-6.6	{ Apparently coprecipitation of CaSO <sub>4</sub>
$1.16 \times 10^{-3} " = 16.8$	20.08	18.2	-9.4	

found a solubility of magnesium picrolonate corresponding to 8.45 mg. per liter at 20° in the undiluted buffer. The supersaturated solutions of magnesium picrolonate are not indefinitely stable. When to 5 ml. of 0.01 M magnesium chloride 1 ml. of buffer and 10 ml. of 0.01 M picrolonic acid were added, some precipitate had appeared the next day (about 24 hours later). Nevertheless even a large excess of magnesium does not interfere in the determination of calcium.

The results of determinations in presence of Na, K, NH<sub>4</sub>, Mg, SO<sub>4</sub>, and PO<sub>4</sub> are given in Table VIII. The samples were kept overnight in a cool room (about 10°) unless otherwise stated.

*Influence of Cations*—The concentrations at which alkali and magnesium interfere depend on the final concentration of these cations in the mixtures and on the excess of picrolonic acid used. In general the concentration of alkali or of magnesium in the sample after addition of buffer and of picrolonic acid may be 0.05 to 0.06 M. Actually the concentration may be greater in many cases (for example in Experiments 3, 14, 25, 26, Table VIII). A 0.01 M calcium solution which was 0.25 M in potassium chloride (Experiment 7) could be determined because the mixture became sufficiently diluted after addition of buffer and picrolonic acid. On the other hand the determination of 0.001 M Ca in 0.25 M KCl (Experiment 10) became highly erratic because of the precipitation of potassium picrolonate. At this small calcium concentration the volume of picrolonic acid added did not dilute the solution sufficiently to prevent precipitation of potassium picrolonate. In Experiments 25 and 26 made in 0.25 M sodium solution the determination of 0.001 M calcium was correct, since the excess of picrolonic acid used was only 15 micromoles, but when the excess was 25 micromoles (Experiment 27) a strong interference by the precipitation of sodium picrolonate was found. This was not the case when the sodium concentration in the beginning was only 0.1 M (Experiment 6). Potassium and ammonium interfere more than magnesium.

The incomplete precipitation from 0.001 M calcium solution at 15° again is shown in Experiment 5 which corresponds to Experiment 12 in Table V. Experiments 22 and 23 again show that the determination of 0.01 M calcium gives somewhat high values when the sample is kept in the ice box overnight.

*Influence of Anions*—Phosphate when present in a concentration of 0.25 M did not interfere. With 0.25 M sulfate a slight interference was found, undoubtedly due to coprecipitation of calcium sulfate (Experiments 30 to 32). Interference did not occur when the sulfate concentration was 0.15 M, which still is a large excess with respect to the calcium concentration.

Quite generally, then, the method yields good results in the determination of 0.001 to 0.01 M calcium solutions even when the concentrations of alkali,

magnesium, sulfate, and phosphate ions are from 10 to 100 times larger than that of the calcium.

### Procedure

Determine the approximate calcium content of the sample by a preliminary test as described above. Add a quantity of buffer solution (1 M acetic acid, 0.125 M lithium acetate, 1 M lithium chloride) such that it becomes about 10 times diluted in the final mixture. Add such an amount of standard 0.01 M picrolonic acid to 5 to 10 ml. of the unknown that the concentration of picrolonic acid after completed precipitation becomes at least 0.001 M. The amount of picrolonic acid added shall not exceed the 4-fold molar concentration of the calcium. Keep the mixtures overnight in an ice box when the calcium concentration is equal to or smaller than  $5 \times 10^{-3}$  M. When the calcium concentration is greater, keep it at room temperature (not above 20°). The residual concentration of picrolonic acid is determined without filtering polarographically at 20° at a cathode potential of -1.1 volts *versus* the saturated calomel electrode or when a mercury pool anode is used at an applied E.M.F. of -1.2 volts. The picrolonic acid solution prepared from the recrystallized product is standardized by titrating with carbonate-free 0.1 M sodium hydroxide with phenolphthalein as indicator.

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### SUMMARY

1. Calcium can be determined in a concentration range of from 0.001 to 0.01 M by precipitation as calcium picrolonate with an excess of standard picrolonic acid solution. The excess of reagent is determined polarographically without filtering the solutions. The method is accurate within 1 to 2 per cent.

2. The method yields good results in the presence of relatively large amounts of Na, K,  $\text{NH}_4$ , Mg, sulfate, and phosphate in the solutions.

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# THE EFFECT OF GLUCOSE FEEDING UPON THE QUANTITATIVE RELATIONSHIP BETWEEN $\beta$ -HYDROXYBUTYRIC ACID AND ACETOACETIC ACID IN BLOOD AND URINE\*

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Ketosis that is caused in healthy individuals by fasting or by a carbohydrate deficiency in the food readily subsides in a few hours after the administration of carbohydrates. When, as a result, ketonuria diminishes,  $\beta$ -hydroxybutyric acid disappears from the urine more rapidly than acetoacetic acid; as Kennaway expressed it (1), the  $\beta$  ratio,  $(100 \times \beta\text{-hydroxybutyric acid})/(\text{total ketone bodies})$ , gradually decreases. This observation of several earlier workers (1, 2) was confirmed in part in our laboratory. Since it is possible, however, that interconversion between acetoacetic acid and  $\beta$ -hydroxybutyric acid may take place in the kidneys, we followed the changes of the  $\beta$  ratio not only in the urine but also in the blood. In view of our observations that in the postabsorptive state the ketone bodies are unevenly distributed between corpuscles and plasma and, furthermore, that the distribution of acetoacetic acid and of  $\beta$ -hydroxybutyric acid shows substantial differences (3), we have studied all of these relationships during the process of recession of ketosis resulting from carbohydrate feeding.

## *Healthy Subjects*

For observations on non-diabetic ketosis, three healthy young men were fasted for 62, 38, and 64 hours, respectively. All of them showed marked ketonuria at the end of the fasting period. At this point blood samples were obtained directly before and at hourly intervals after the administration of 100 gm. of glucose. The ketone bodies were determined in whole blood and in plasma by a procedure outlined in a preceding publication (3). The values for the corpuscles were calculated from these analytical data and the cell volume.

The data presented in Table I show that, as previously reported (3), the corpuscles contain considerably smaller amounts of ketone bodies than does the plasma; in our three subjects the concentration in the plasma was from 2 to 3 times as high as in the cells. The administration of glucose, however, effected changes in the distribution. Thus in Subject 1 the distribution ratio (ketone body concentration in the cells)/(ketone body concentration in the plasma), which in the fasting state was 0.39, rose to 1.06

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during the 3rd, and to 4.50 during the 4th hour after glucose feeding; that is to say, the ratio was inverted, and the concentration of ketone bodies in the corpuscles ultimately increased to more than 4-fold the concentration in the plasma. Subjects 2 and 3 showed changes of much the same character.

We interpret this fact as follows: Hyperketonemia occurs when the liver transmits ketone bodies into the blood stream at a higher rate than the extrahepatic tissues are able to consume them. After glucose feeding the liver begins to catabolize carbohydrate in preference to fat and protein, so that the production of ketone bodies is gradually arrested. In consequence the blood receives from the liver smaller amounts of ketone bodies than it gives up to the extrahepatic tissues (and to some extent to the urine),

TABLE I

*Effect of Glucose Feeding upon Distribution of Total Ketone Bodies between Corpuscles and Plasma in Normal Subjects*

Subject No.	Time since last meal	Time after glucose feeding	Total ketone bodies in			Ketones in corpuscles Ketones in plasma
			Whole blood	Corpuscles	Plasma	
	hrs.	hrs.	mg. per cent	mg. per cent	mg. per cent	
1	62	0	23.1	13.1	33.7	0.39
		1	21.5	14.1	28.8	0.49
		2	5.80	5.63	5.95	0.95
		3	1.61	1.65	1.55	1.06
		4	0.58	0.90	0.20	4.50
2	38	0	17.9	9.24	26.0	0.36
		1	9.31	5.66	13.2	0.43
		2	3.57	3.50	3.56	1.00
		3	2.51	3.32	1.43	2.32
		4	1.53	2.20	0.42	5.20
3	64	0	68.4	48.8	87.9	0.56
		1	58.2	47.3	70.0	0.67
		3	17.2	18.3	16.4	1.11

and the ketonemic level recedes. As a matter of course this change affects primarily the concentration of plasma ketones, and thereby, secondarily, induces a diffusion of ketone bodies from cells to plasma. The diffusion apparently lags behind the rate of decrease in the plasma and, as a result, the distribution ratio shifts in favor of the corpuscles.

In the same three subjects we also examined the possible effect of glucose feeding upon the relationship between  $\beta$ -hydroxybutyric acid and acetoacetic acid ( $\beta$  ratio) both in corpuscles and in plasma. The results, given in Table II, show a continual decline of the  $\beta$  ratio in the plasma as well as in the corpuscles. It has markedly decreased already during the 1st hour after the administration of glucose, and in the 3rd and 4th hours it dropped

to zero. This means the complete disappearance of  $\beta$ -hydroxybutyric acid at these periods; so that the ketone bodies in the blood were represented solely by acetoacetic acid both in corpuscles and plasma. The complete disappearance of  $\beta$ -hydroxybutyric acid occurred in the corpuscles earlier than in the plasma in Subjects 2 and 3, but later in Subject 1. An interesting fact is that, whereas the concentration of  $\beta$ -hydroxybutyric acid in the plasma usually remained higher than in the cells, the concentration

TABLE II

*Effect of Glucose Feeding upon Distribution of Acetoacetic and  $\beta$ -Hydroxybutyric Acids in Blood of Normal Subjects*

Subject No.	Time after glucose	Corpuscles				Plasma			
		Acetoacetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio	Acetoacetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio
	hrs.	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
1*	0	4.08	9.01	13.1	70	5.47	28.2	33.7	84
	1	6.75	7.32	14.1	52	7.02	21.8	28.8	76
	2	3.95	1.68	5.63	30	2.65	3.30	5.95	56
	3	0.98	0.67	1.65	40	1.55	0	1.55	0
	4	0.90	0	0.90	0	0.20	0	0.20	0
2†	0	5.32	3.92	9.24	43	8.36	17.6	26.0	68
	1	5.66	0	5.66	0	5.51	7.60	13.2	58
	2	3.18	0.32	3.50	9	2.86	0.70	3.56	20
	3	3.32	0	3.32	0	1.43	0	1.43	0
	4	2.20	0	2.20	0	0.42	0	0.42	0
3‡	0	26.1	22.7	48.8	42	21.8	66.1	87.9	75
	1	27.3	20.0	47.3	42	29.5	40.5	70.0	60
	3	18.3	0	18.3	0	13.3	3.1	16.4	19
4§	0					0.25	0.70	0.95	74
	2					0.12	0.10	0.22	45
5§	0					0.11	0.27	0.38	71
	2					0.24	0.11	0.35	33

\* Fasted 62 hours.

† Fasted 38 hours.

‡ Fasted 64 hours.

§ In postabsorptive state, 14 hours after last meal.

of acetoacetic acid tended to shift in favor of the corpuscles, especially in the 3rd and 4th hours after glucose feeding. In Subjects 1 and 2, for instance, the acetoacetic acid in the corpuscles eventually became from 2 to 5 times as high as in the plasma. A possible cause of this phenomenon is that the cells tend to retain acetoacetic acid more firmly than  $\beta$ -hydroxybutyric acid under conditions that cause a rapid decline in the ketone body concentration of the plasma.



As regards Subjects 4 and 5 in Table II, these were normally nourished healthy individuals in the postabsorptive state (14 hours after the last meal) when subjected to the experiment. In view of the large quantities of blood required for analysis in such cases, only two samples were taken, one before and one 2 hours after glucose feeding. We have previously described the observation that glucose feeding measurably depresses the ketonemic level of the well nourished healthy individual just as it does in the instance of fasting ketosis (4). Subjects 4 and 5 show that the response of the two groups is similar also in regard to changes in the  $\beta$  ratio. Even in Subject 5, whose basal ketonemic level was so low (0.38 mg. per cent) that its probable decrease after glucose feeding may have eluded measure-

TABLE III

*Effect of Glucose Feeding on Relationship between Acetoacetic Acid and  $\beta$ -Hydroxybutyric Acid in Urine of Normal Subjects*

Subject No.	Time after glucose	Acetoacetic acid		$\beta$ -Hydroxybutyric acid		Total ketone bodies		$\beta$ ratio
		mg. per cent	mg. per hr.	mg. per cent	mg. per hr.	mg. per cent	mg. per hr.	
1	0	96.5	20.7	131.0	28.0	227.5	48.7	58
	1	129.0	36.3	85.0	23.8	214.0	64.1	40
	2	38.5	8.86	0	0	38.5	8.86	0
	3	5.90	1.89	5.12	1.64	11.0	3.52	46
	4	2.95	0.74	5.58	1.40	8.53	2.14	71
2	0	30.6	11.5	74.7	28.0	105.3	39.5	72
	1	16.5	6.61	15.1	6.05	31.6	12.7	48
	2	5.73	1.60	0	0	5.73	1.60	0
	3	3.33	7.33	1.43	3.58	4.76	10.9	31
	4	3.00	6.90	0.84	1.93	3.84	8.83	22
3	0	70.0	58.1	149.0	124.0	219.0	182.1	68
	1	15.5	8.27	13.6	7.27	29.1	15.5	47
	2.5	91.7	149.2	0	0	91.7	149.2	0

ment, the drop in the  $\beta$  ratio was still great enough to be detected. In Subject 4 the ketonemic level was high enough (0.95 mg. per cent) for reliable measurement; here it is clearly shown that the  $\beta$  ratio decreased materially as the ketonemic level was depressed by glucose feeding, in the same manner as in fasting ketosis.

Changes in the relationship between acetoacetic acid and  $\beta$ -hydroxybutyric acid were examined at hourly intervals in urine samples which were collected simultaneously with the blood samples. The results, given in Table III, show that the  $\beta$  ratio declined in urine faster than in the blood. This tendency was evident as early as in the 1st hour, and quite conspicuous during the 2nd hour after glucose feeding; at this time  $\beta$ -hydroxybutyric

acid completely disappeared in the urine, which was 1 hour earlier than in the blood in all three of our fasted subjects. The difference between blood and urine was further accentuated when  $\beta$ -hydroxybutyric acid emerged again in the urine during the 3rd and 4th hours, when it was completely absent from the blood.

TABLE IV

*Effect of Glucose Feeding upon Distribution of Total Ketone Bodies between Corpuscles and Plasma in Diabetic Subjects*

Subject No.	Time after glucose feeding	Total ketone bodies in			Ketones in corpuscles Ketones in plasma
		Whole blood	Corpuscles	Plasma	
	<i>hrs.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
6	0	25.4	8.49	38.7	0.22
	0.5	41.0	28.6	49.8	0.57
	1	35.5	21.9	50.6	0.43
	2	33.2	18.7	44.4	0.42
	3	38.3	48.3	29.8	1.62
	4	43.1	36.5	49.8	0.73
7	0	68.0	60.2	78.8	0.76
	1.5	82.0	67.7	95.4	0.71
	3.5	101.3	95.9	105.6	0.91
8	0	4.78	4.78	4.78	1.00
	1	3.98	1.53	6.10	0.25
	2	4.04	4.26	3.80	1.12
	3	4.16	2.36	6.10	0.39
	4	3.67	2.90	4.40	0.66
9	0	63.8	52.9	71.9	0.74
	0.5	62.9	25.9	87.6	0.29
	1	70.7	66.4	73.7	0.90
	2	58.0	37.6	70.7	0.53
	3	55.3	31.3	72.1	0.43
	4	43.0	20.8	56.8	0.37

### *Diabetic Subjects*

On the basis of previous studies we divide diabetic subjects, with respect to their response to glucose feeding, into two main categories. Those in the first exhibit a tendency towards rising ketonemic levels; those in the second show a rather consistent decrease of ketonemia. Patients belonging to the latter group behave much like the healthy individuals whose ketosis resulted from fasting or a carbohydrate-deficient diet. In a previous paper, this difference was attributed to differences in the liver function of the patients (5).

Tables IV to VI contain results of observations on representative cases of the first category. As may be seen in Table IV, the distribution of total

ketone bodies between corpuscles and plasma showed changes quite different from those observed in healthy individuals (*cf.* Table I); *i.e.*, instead of a consistent increase of the distribution ratio in favor of the corpuscles, considerable and rather irregular fluctuations occurred. In Subject 7 the ratio showed a tendency to increase at the end, somewhat as in healthy subjects, but in Subjects 8 and 9 the opposite tendency was in evidence during the 3rd and 4th hours after glucose feeding.

TABLE V

*Effect of Glucose Feeding upon Distribution of Acetoacetic and  $\beta$ -Hydroxybutyric Acids in Blood of Diabetic Subjects*

Subject No.	Time after glucose	Corpuscles				Plasma			
		Aceto-acetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio	Aceto-acetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio
	hrs.	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
6	0	8.47	0.02	8.49	0.2	7.20	31.5	38.7	81
	0.5	12.4	16.1	28.6	57	9.10	40.7	49.8	82
	1	4.87	17.0	21.9	78	14.3	36.3	50.6	72
	2	10.0	8.69	18.7	47	11.8	32.6	44.4	73
	3	9.00	39.3	48.3	81	9.00	20.8	29.8	70
	4	11.9	24.5	36.5	67	15.3	34.5	49.8	69
7	0	29.1	31.1	60.2	52	19.1	59.8	78.8	76
	1.5	39.2	28.5	67.7	42	23.5	71.9	95.4	75
	3.5	54.5	41.4	95.9	43	23.8	81.8	105.6	63
8	0	1.31	3.47	4.78	73	1.31	3.47	4.78	73
	1	0.43	1.20	1.63	62	1.40	4.70	6.10	78
	2	1.76	2.50	4.26	59	0.80	3.00	3.80	78
	3	1.20	1.16	2.36	49	1.70	4.40	6.10	74
	4	1.04	1.86	2.90	64	1.20	3.10	4.40	72
9	0	23.3	29.6	52.9	56	21.2	50.7	71.9	71
	0.5	10.7	15.2	25.9	59	24.9	62.7	87.6	71
	1	27.1	39.3	66.4	59	21.5	52.2	73.7	71
	2	22.7	14.9	37.6	40	20.3	50.4	70.7	71
	3	10.9	20.4	31.3	65	21.7	50.4	72.1	70
	4	8.30	12.5	20.8	60	16.1	40.7	56.8	72

Likewise, the response was entirely different from the normal as regards the changes in the relationship between  $\beta$ -hydroxybutyric acid and acetoacetic acid. As may be noted in Table V, the  $\beta$  ratio showed at no time any appreciable decrease either in the corpuscles or in the plasma, and the actual amount of  $\beta$ -hydroxybutyric acid tended to increase at such periods at which it completely disappeared in non-diabetic ketosis. The maintenance of high  $\beta$  ratios was more consistent in the plasma than in the corpuscles.

The difference between this group of diabetic patients and healthy individuals was just as marked in the urine as in the blood. As it may be seen in Table VI, the relative quantities of  $\beta$ -hydroxybutyric acid have not appreciably changed in the course of the entire 4 hours after glucose feeding. During the 2nd hour, when  $\beta$ -hydroxybutyric acid had completely disappeared from the urines of healthy subjects (*cf.* Table III), the three diabetic subjects showed  $\beta$  ratios of 73, 70, and 82, respectively, values of the same order of magnitude as one finds in fasting or in postabsorptive states.

Diabetic patients belonging to the second category, who respond to glucose feeding with a progressive decline of the ketonemic level, as do

TABLE VI

*Effect of Glucose Feeding on Relationship between Acetoacetic Acid and  $\beta$ -Hydroxybutyric Acid in Urine of Diabetic Subjects*

Subject No.	Time after glucose	Acetoacetic acid		$\beta$ -Hydroxybutyric acid		Total ketone bodies		$\beta$ ratio
		mg. per cent	mg. per hr.	mg. per cent	mg. per hr.	mg. per cent	mg. per hr.	
6	0	55.0		120.0		175.0		69
	1	28.1	45.6	96.2	156.0	124.0	201.6	77
	2	16.5	43.4	44.0	116.3	60.5	159.7	73
	3	16.6	78.5	43.8	206.7	60.4	285.2	73
	4	20.0	78.8	118.0	460.4	138.0	539.2	85
7	0	48.6		167.0		215.3		78
	1	54.0	184.0	139.0	471.0	193.0	655.0	72
	2	53.0	249.0	123.0	579.0	176.0	829.0	70
	3	40.0	209.0	129.0	672.0	169.0	881.0	76
	4	50.0	111.0	131.0	287.0	181.0	398.0	73
10	0	47.0	47.0	145.0	145.0	192.0	192.0	76
	1	53.0	127.0	187.0	448.0	240.0	575.0	78
	2	68.0	60.6	298.0	269.0	366.0	329.0	82
	3	65.0	51.9	278.0	223.0	343.0	275.0	76
	4	109.0	65.5	371.0	223.0	480.0	287.0	77

healthy men (although at a lower rate), also show tendencies in the direction of normal as regards the changes in the relationship between  $\beta$ -hydroxybutyric acid and acetoacetic acid, both in blood and urine. This fact is illustrated by an example, Subject 11, in Table VII. As may be seen, in the blood the  $\beta$  ratio began to decrease during the 2nd hour after glucose feeding, and continued to decline throughout the 4 hour period. This decrease was more delayed and distinctly less extensive than in non-diabetic subjects. A similar change has occurred in the urine; namely, the  $\beta$ -hydroxybutyric acid has at no time disappeared as in fasting ketosis, and yet the  $\beta$  ratio dropped from the postabsorptive level of 70 to 53 during the 1st hour after glucose feeding. This drop was then followed by an

increase during the remainder of the 4 hour period (*cf.* Table III). It may be stated then that in this group of diabetic individuals glucose feeding elicits changes both in ketonemia and ketonuria that show the same tendency as the changes in healthy subjects. There is only a quantitative difference, in that the changes in the diabetic proceed at a diminished rate.

Subject 12 was included in Table VII to emphasize further the great differences between blood and urine in the changes in the  $\beta$  ratio after glucose feeding. This individual belongs to the first category of diabetics; namely, to the group that does not respond with a consistent decrease in

TABLE VII

*Comparison of Effect of Glucose Feeding on Changes of  $\beta$  Ratio in Blood and Urine of Diabetic Subjects*

Subject No.	Time after glucose	Whole blood				Urine			
		Aceto-acetic acid	$\beta$ hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio	Aceto-acetic acid	$\beta$ hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio
	hrs.	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
11	0	2.60	8.40	11.0	76	*	*	*	70
	0.5	2.00	4.46	6.45	69	47.4	70.6	118.0	70
	1	2.00	5.30	7.30	76	13.5	30.5	57.4	53
	2	1.60	2.90	4.50	64	28.0	32.9	60.9	55
	3	1.50	3.00	4.50	67	17.4	37.8	55.1	69
	4	1.90	2.80	4.70	60	11.9	17.8	29.7	60
12	0	1.63	5.17	6.80	76	*	*	*	36
	0.5	1.12	3.80	4.92	77				
	1	1.15	4.17	5.32	78	3.88	0.05	3.93	1
	2	1.59	5.17	6.76	76	11.1	0.64	11.7	6
	3	1.59	4.66	6.25	75	4.34	1.98	6.32	31
	4	1.14	3.05	4.19	73	4.54	4.72	9.26	51

\* The interval of time during which the specimen was excreted is unknown; hence the rate of excretion of ketone bodies could not be calculated. The concentration of acetoacetic acid and  $\beta$ -hydroxybutyric acid at 0 hour was as follows: in Subject 11, 25.0 and 57.3 mg. per cent; in Subject 12, 7.71 and 6.32 mg. per cent.

the ketonemic level. As in other cases of this group, the  $\beta$  ratio in the blood remained virtually unaltered during the 4 hour period after the administration of glucose. The changes in the urine, on the other hand, were entirely unexpected. Here the  $\beta$  ratio (which was already exceptionally low in the postabsorptive state) dropped steeply in the first 2 hours and then increased, much as in the fasting ketosis of healthy subjects.

The divergence between the changes of the  $\beta$  ratio in the blood and urine after glucose feeding was conspicuous enough in our non-diabetic subjects (*cf.* Table III). It is evidently due to a reversible conversion between

acetoacetic acid and  $\beta$ -hydroxybutyric acid, which has been known to occur in the kidney. Subject 12 (Table VII) vividly illustrates how extensive this effect of the kidney can be. It is a warning against the injudicious use of data of urine analysis as the basis of conclusions concerning the changes of the ketone bodies in the living organism. To explore these changes one must resort to the analysis of blood and other tissues.

The most notable change in the blood, recorded in this report, is the rapid decrease and eventual complete disappearance of  $\beta$ -hydroxybutyric acid<sup>1</sup> while measurable amounts of acetoacetic acid are still present. This occurs when glucose feeding exerts its fat- (and protein-) sparing effect in the liver and, in consequence, the liver ceases to produce and transmit to the blood excessive amounts of ketone bodies. In other words, two facts, namely the suppression of hepatic ketone production and the disappearance of  $\beta$ -hydroxybutyric acid from the blood, appear to be in a simple causal connection, implying that the ketone body supplied by the liver is mainly, if not exclusively,  $\beta$ -hydroxybutyric acid. This inference is at variance with the widely held view that the parent ketone body formed in the tissues is acetoacetic acid. In our opinion no evidence presented thus far to support either of the two conflicting views can be regarded as conclusive and unequivocal.

#### SUMMARY

In postabsorptive states the ratio of  $\beta$ -hydroxybutyric acid to acetoacetic acid in blood and urine, as also of the distribution of the two acids between corpuscles and plasma, is independent of the ketonemic level; in this respect no difference exists between healthy and diabetic subjects. Glucose feeding elicits the following changes.

1. In the blood of healthy persons, in the state of hunger ketosis, the concentration of ketone bodies decreases faster in the plasma than in the corpuscles; this leads to an inversion of the distribution ratio (total ketone bodies in corpuscles)/(total ketone bodies in plasma), which in the post-absorptive state is below unity.

2. The  $\beta$  ratio ( $100 \times \beta$ -hydroxybutyric acid)/(total ketone bodies) decreases consistently and drops to zero during the 3rd and 4th hours after glucose feeding, so that at these intervals the ketone bodies are represented by acetoacetic acid alone. This change proceeds faster in the plasma than in the corpuscles.

<sup>1</sup> It is in order, perhaps, to remark here that the disappearance of  $\beta$ -hydroxybutyric acid can be demonstrated only by analytical methods that are sufficiently selective for acetone. By simply measuring the mercury content of the Denigès precipitate, as for example in Crandall's technique (6), one always obtains positive values for  $\beta$ -hydroxybutyric acid.

3. In the urine of these subjects the  $\beta$  ratio decreases more rapidly than in the blood and the  $\beta$ -hydroxybutyric acid completely disappears about an hour earlier than in the blood. In contrast to the blood, however, it emerges again at an increasing rate during the 3rd and 4th hours.

4. Diabetic patients, whose livers have retained to an appreciable degree the ability to utilize carbohydrate, respond to glucose feeding as do healthy individuals, with a difference only in the rate of the process.

5. Diabetic patients of another category, i.e. those whose livers are unable to utilize appreciable amounts of carbohydrate and, in consequence, maintain (or more often increase) their ketonemic level after glucose feeding, show no significant changes in the blood. The distribution ratio between corpuscles and plasma, as well as the  $\beta$  ratio, remains substantially the same as that in the postabsorptive state. As a rule, this applies also to urines of these patients, but there are exceptions.

6. The frequent divergence between the changes in blood and urine, due to oxidation-reduction processes in the kidney, suggests that studies of the metabolism of ketone bodies should include analyses of blood and of other tissues, since data derived from the urine alone may be misleading.

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# THE EFFECT OF INSULIN UPON THE QUANTITATIVE RELATIONSHIP BETWEEN $\beta$ -HYDROXYBUTYRIC ACID AND ACETOACETIC ACID IN BLOOD AND URINE\*

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In a preceding report (1) we described cases of diabetic ketosis in which glucose feeding fails to produce appreciable changes either in the distribution of ketone bodies between blood corpuscles and plasma or in the  $\beta$  ratio ( $100 \times \beta$ -hydroxybutyric acid)/(total ketone bodies) of blood and urine. These subjects, in contrast to those with non-diabetic ketosis, tend to respond to glucose feeding with an increase of the ketonemic level and of ketonuria and require massive doses of insulin for the suppression and abolition of ketosis. Their condition is attributed to the impaired ability of the liver to store glycogen and to burn carbohydrate, a deficiency that can be corrected only by the administration of adequate amounts of insulin which acts by inhibiting hepatic glycogenolysis (2). The purpose of our experiments was to compare the effects of insulin (combined with carbohydrate feeding) upon the distribution and  $\beta$  ratios in this type of diabetic ketosis with the effects of glucose feeding in fasting ketosis.

The insulin requirement in severe diabetic ketosis is always large; individual variations, however, are considerable and unpredictable, so that overinsulinization may entail hypoglycemic episodes. Since hypoglycemia causes an increase in ketosis, and thus reverses the antiketogenic effect of insulin (3), we were careful to forestall hypoglycemia by always keeping the blood sugar well above normal postabsorptive levels by timely administration of glucose. At the same time care was taken to maintain a positive balance between carbohydrate administered and glucose lost in the urine in order to ascertain that substantial amounts of carbohydrate were utilized by the organism.

Under these conditions the distribution of ketone bodies between corpuscles and plasma as well as the  $\beta$  ratio changed in diabetic patients in the same general direction as in non-diabetic subjects after glucose feeding (without insulin injections). Evidence to illustrate this fact is presented in Table I. As regards the distribution of ketone bodies between corpuscles and plasma in the postabsorptive state, it may be seen that, as in healthy individuals, the concentration was markedly higher in the plasma than in the corpuscles. But after glucose utilization had started, within some

\* This work was aided by the Helen Yonkers Research Fund.



hours after the injection of insulin, the distribution ratio shifted in favor of the cells; i.e., the decline was faster in the plasma than in the cells, just as in non-diabetic ketosis. This response was considerably slowed down in the instance of patient L. L., in deep coma, who was much more resistant to insulin action than were the other two patients.

The  $\beta$  ratio, as may be noted, decreased substantially after insulin action had taken effect; in other words, the concentration of  $\beta$ -hydroxybutyric acid decreased faster than the concentration of acetoacetic acid. The shift in the relationship between the two acids was more prominent in the

TABLE I

*Effect of Insulin upon Distribution of Acetoacetic Acid and  $\beta$ -Hydroxybutyric Acid in Blood of Diabetic Subjects*

Patient and remarks	Time after insulin	Corpuscles				Plasma			
		Acetoacetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio	Acetoacetic acid	$\beta$ -Hydroxybutyric acid	Total ketone bodies	$\beta$ ratio
	hrs	mg. per cent	mg. per cent	mg. per cent		mg. per cent	mg. per cent	mg. per cent	
L. L. Admitted in coma; received 200 units insulin during first 4 hrs. after admission	0	63.4	82.9	142.3	57	92.8	84.3	177.0	48
	6	43.1	58.4	101.5	58	115.0	93.9	209.0	45
	13	32.6	3.21	35.8	9	21.2	3.22	24.4	13
	19	14.7	3.00	17.7	17	9.28	2.98	12.3	24
M. R. Severe ketosis; received 45 units insulin on admission	0	40.0	48.3	88.2	55	44.4	98.6	143.0	69
	2	52.6	28.2	80.8	35	40.5	41.2	81.7	51
	6					30.0*	4.70*	34.7*	13*
S. B. Severe ketosis; received 55 units insulin during 4 hrs. after admission	0					57.6*	93.7*	151.0*	66*
	6.5	60.0	14.0	74.0	19	40.8	22.6	63.4	36

\* These data represent the analysis of whole blood; the samples were not sufficient for the analysis of the plasma.

corpuscles than in the plasma, especially in the more advanced stages of the process. These changes are of the same character as those observed in healthy subjects, with the sole difference that they proceed at a lower rate in diabetic ketosis, and in particular in the state of coma. It is due to this diminished rate that in no case of diabetic ketosis has the complete disappearance of  $\beta$ -hydroxybutyric acid been observed, whereas, in non-diabetic ketosis this occurs within 3 to 4 hours after glucose feeding.

These changes in diabetic and non-diabetic ketosis then are due to the same cause; namely, to the fat- and protein-sparing effect of carbohydrate

in the metabolism of the liver. Ketosis in the diabetic organism is due to an unbridled hepatic glycogenolysis. Hand in hand with the inability of the liver to store glycogen goes its inability to use carbohydrate to cover its own fuel requirement. As a result, fats and proteins are used and abnormal amounts of ketone bodies are transmitted to the blood. Then, when insulin begins to inhibit hepatic glycogenolysis (besides enhancing the passage of carbohydrate into muscle cells), the liver is again in a position to burn its preferential fuel, carbohydrate, and the oxidation of excessive amounts of fats and proteins, the source of ketone bodies, is suppressed. This is the same process that occurs in fasting ketosis as the result of carbohydrate feeding alone.

Changes in the urines of diabetic patients, when ketonemia decreases under the action of insulin, are illustrated in Table II which contains the results of observations on two patients whose blood also had been studied. Urine samples were collected at brief intervals of time, without the loss of any fraction that was excreted over the entire period of the observations. It may be noted in both cases that after insulin action had become effective (as evidenced by utilization of carbohydrate) ketonuria began to diminish rapidly. Simultaneously the  $\beta$  ratio showed a moderate initial decrease. But whereas in fasting ketosis the  $\beta$  ratio was reduced to zero within 2 hours after glucose feeding (*i.e.*,  $\beta$ -hydroxybutyric acid completely disappeared from the urine), in the two diabetic patients it has not dropped below 55 and 60, respectively. After this initial decline the  $\beta$  ratio rose again and reached the preinsulin level, despite the fact that at the same time ketonuria continued to decrease substantially. The  $\beta$  ratio, then, changed here in the same manner as in fasting ketosis, with only a quantitative difference, inasmuch as the changes were less extensive and proceeded at a diminished rate.

In urine samples that were collected after the period in which the  $\beta$  ratio had returned to the high basic level, a second drop occurred, which was considerably greater than the first. In both patients M. R. and S. B. the second drop has taken place during the 7th hour after the first insulin injection, the  $\beta$  ratio having decreased in the two cases to as low as 23 and 33, respectively. This drop was again followed by a rise, notwithstanding the fact that the rate of excretion of total ketone bodies has not increased. We consistently obtained similar results in studies of several other diabetic patients.

These findings are at variance with the view (4) that the  $\beta$  ratio in the urine decreases whenever the rate of excretion of ketone bodies decreases. The fact seems to be that an initial decrease of the  $\beta$  ratio is followed by an increase and subsequently by another decrease. This is equally true for fasting ketosis under the influence of glucose feeding and for diabetic

ketosis under the influence of insulin action. In our study of fasting ketosis, however, we could record only one drop and the subsequent increase of the  $\beta$  ratio, because the experiments were confined to 4 hour periods.

TABLE II

*Effect of Insulin upon Relationship between Acetoacetic Acid and  $\beta$ -Hydroxybutyric Acid in Urine of Diabetic Subjects*

Patient	Time	Remarks	Vol- ume	Glucose	Aceto- acetic acid	$\beta$ - Hydroxy- butyric acid	Total ketone bodies	$\beta$ ratio
			cc	gm per hr	mc per hr	mc. per hr.	mg. per hr.	
M. R.	4.00- 5.30	Vomited 4-5 times be- fore insulin was given	570	13.3	510.0	1824.0	2234.0	77
	5.30- 7.00	25 units insulin at 5.30	330	6.6	138.0	851.0	1289.0	66
	7.00- 7.45	20 " " " 7.30	100	5.3	275.0	333.0	606.0	55
	7.45- 8.30	15 gm. glucose intra- venously at 7.45	65	1.7	130.0	449.0	579.0	68
	8.30-10.00	15 units insulin at 9.05, 24 gm. CHO at 9.30	115	0.5	72.7	160.0	233.0	69
	10.00-11.00	5 units insulin and 28.5 gm. CHO at 10.15	45	0	9.60	33.0	42.6	77
	11.00-12.00	57 gm. CHO at 11.00	75	0	8.40	2.10	10.4	23
	12.00- 2.00	66 " " " 1.00	110	0	11.5	5.10	16.6	31
	2.00- 3.00		200	0	34.1	5.50	39.6	14
	3.00- 5.00	33 " " " 3.05	775	0	47.4	6.20	53.7	21
S. B.	5.00- 7.00		135	0	4.50	6.50	11.0	59
	10.30-12.30	40 units insulin at 11.30	450	11.3	236.5	951.0	1187.0	80
	12.30- 1.30	24 gm. CHO at 1.30	450	13.5	795.0	1292.0	2087.0	62
	1.30- 2.30	18 " " " 2.00	450	5.8	663.0	995.0	1658.0	60
	2.30- 3.30	15 units insulin at 3.00	320	11.2	426.0	703.0	1129.0	62
	3.30- 4.30		140	5.6	108.0	203.0	311.0	65
	4.30- 5.30	28.5 gm. CHO at 5.00	85	0.9	25.6	69.3	94.9	73
	5.30- 6.30	28.5 " " " 6.05	190	0	43.7	21.9	65.6	33
	6.30- 8.00	48 gm. CHO at 6.45	165	0.6	25.6	59.0	85.6	69
	8.00- 9.00	74 " " " 9.00	280	2.8	17.6	73.9	91.5	81
	9.00-10.00	25 " " " 9.10	275	11.0	31.1	32.2	63.3	51
	10.00-11.00		300	15.0	70.5	49.2	119.7	41
	11.00-12.00	24 " " " 11.40	175	8.8	39.2	20.7	59.9	35

The waves in which preponderance of  $\beta$ -hydroxybutyric acid alternates with the preponderance of acetoacetic acid can be observed only when urine samples are collected at brief (1 to 2 hour) intervals of time. Collection of samples during longer periods necessarily blurs the picture. With the example presented in Table III we wish to demonstrate how misleading the analytical data of urines can be if this fact is ignored. In the instance

of patient L. G. blood samples, which were taken at 0, 6, and 10.5 hours after the first injection of insulin, showed the regularly recurrent picture; namely, a consistent decrease of the  $\beta$  ratio when the ketonemic level declined. Urine samples which were excreted during 1 hour periods coincident with the sampling of blood would indicate that the changes in the urine ran parallel with the changes in the blood. However, the analysis of intermediate hourly urine samples, presented in parentheses in Table III, clearly shows the wave-like fluctuations of the  $\beta$  ratio in the urine.

TABLE III

*Effect of Insulin on Ketone Bodies in Whole Blood and Urine of Diabetic Subject*

L. G., patient admitted in coma; received 285 units of insulin during the first 5 hours following admission. The parentheses designate intermediate hourly urine samples.

Time after insulin	Blood sugar	Whole blood				Urine	
		Aceto-acetic acid	$\beta$ -Hydroxy-butyric acid	Total ketone bodies	$\beta$ ratio	Total ketone bodies	$\beta$ ratio*
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent		mg. per hr.	
0	822	99.0	198.0	297.0	64	791†	76
(2.5)						(1800)	(57)
(3.5)						(2474)	(56)
6	520	98.0	68.0	166.0	41	131	74
7.5	390	80.0	31.0	111.0	28	62.9	54
10.5	244	44.2	2.80	47.0	6	17.7	40

\* These  $\beta$  ratios were determined on specimens excreted during 1 hour periods.

† This is a postabsorptive sample collected at the time of admission; since the length of time during which it was excreted was unknown, the value 791 represents mg. per cent and not mg. per hour.

## SUMMARY

In severe ketosis of diabetic patients glucose feeding has no effect upon the quantitative relationship of ketone bodies in blood and urine. When such patients are enabled to utilize carbohydrate (circulating endogenous glucose or glucose administered either parenterally or orally) by the injection of adequate amounts of insulin, the ketone bodies show changes of the same general nature as those in healthy subjects after glucose feeding. As the ketonemic level decreases, the distribution between corpuscles and plasma shifts in favor of the corpuscles, and the  $\beta$  ratio decreases gradually and consistently. The decrease is faster in the plasma than in the corpuscles.

In the urine the  $\beta$  ratio first decreases, then increases, as it does in non-diabetic persons. Extension of the observations over sufficiently long

periods of time (8 to 12 hours), however, reveals the fact that after an initial decrease the  $\beta$  ratio again rises and then drops for a second time, and this wave-like change repeats itself irrespective of the fact that the excretion of total ketone bodies diminishes. This finding is at variance with the view of previous workers according to which the  $\beta$  ratio always decreases when ketonuria diminishes.

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# FURTHER PURIFICATION OF CATECHOLASE (TYROSINASE)

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In a previous communication (1) we described a method for the preparation from the common mushroom, *Psalliota campestris*, of a catecholase fraction, containing about 500 catecholase units per mg. of dry organic weight. 1 unit of catecholase activity was defined, according to Adams and Nelson (2), as the amount of enzyme required to cause an oxygen uptake of 10 c.mm. per minute when the catechol-hydroquinone substrate was used. We wish to describe briefly a method of further purification of the enzyme by fractional precipitation with ammonium sulfate at pH 4.8 to 4.9 and at a protein concentration of 0.5 per cent.

## EXPERIMENTAL

To the dialyzed enzyme solution, obtained according to the method previously described (1), containing 500 Adams and Nelson catecholase units (2) per mg. of dry organic weight, 1 or 2 drops of saturated sodium hydroxide solution were added, and the pH was readjusted to 4.8 to 4.9 by the addition of glacial acetic acid. Any precipitate formed was removed by centrifugation, and the supernatant was brought to 0.25 saturated (cf. (1)) ammonium sulfate and allowed to stand overnight at room temperature. The precipitate was centrifuged off, and the supernatant brought to 0.35 saturated ammonium sulfate. This precipitation between 0.25 and 0.35 saturated ammonium sulfate was repeated until only a small amount of material precipitated up to 0.25 saturation, and until very little material was left in the supernatant of the 0.25 to 0.35 saturated ammonium sulfate precipitate. The final 0.25 to 0.35 saturated ammonium sulfate precipitate was dissolved in water and dialyzed until free from inorganic salts.

The enzyme preparation thus obtained was found to contain about 1200 Adams and Nelson catecholase units and 48 cresolase units per mg. of dry organic weight. 1 unit of cresolase activity is defined as the amount of enzyme required to cause an oxygen uptake of 10 c.mm. per minute when acting on 4 mg. of *p*-cresol (3). The copper content, determined according to the method of Warburg (4) as modified by Nelson and Dills,<sup>1</sup> was found to be 0.20 per cent.

<sup>1</sup> Nelson, J. M., and Dills, W. L., private communication.

## SUMMARY

A method for the further purification of the enzyme, catecholase (tyrosinase), is described. The purified enzyme preparation was found to contain about 1200 Adams and Nelson catecholase units and 48 cresolase units per mg. of dry organic weight. The copper content was found to be 0.20 per cent.

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# STUDIES RELATING TO THE USE OF *LACTOBACILLUS CASEI* IN MICROBIOLOGICAL ASSAYS

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It has been shown by several groups of investigators (1-4) that the amount of acid or the degree of turbidity developed by *Lactobacillus casei* during an incubation period is proportional to the amount of riboflavin, pantothenic acid, or certain other vitamins in the various media which are described. However, recent studies (5, 6) have demonstrated that under certain conditions more than twice the quantity of acid formerly regarded as maximal can be produced. This would indicate either that some ingredients of the original media were not present in adequate quantity, or that an additional growth substance was being added, or that both of these factors were operating. The purpose of the present work was to investigate the culture media and to study the characteristics of the dose-response curve obtained with graded amounts of pantothenic acid.

## EXPERIMENTAL

The procedure of culturing *Lactobacillus casei*, American Type Culture Collection (No. 7469), and the method of inoculating series of tubes containing the media were those described by Pennington, Snell, and Williams (3).

After an incubation period of 70 to 72 hours, unless otherwise designated, the acid in each tube was determined by titrating to neutrality with 0.1 N NaOH, brom-thymol blue indicator or a pH meter being used.

True turbidity measurements (*i.e.* of light reflected at right angles from the incident beam) were made by means of a photoelectric colorimeter<sup>1</sup> fitted with a brown filter to nullify the color of the cultures. The 18 × 150 mm. test-tubes used in the assays were employed directly in the colorimeter. Calibration of this instrument with standard barium sulfate suspension (7) showed that there was a linear relation between the logarithm of the cc. of barium sulfate suspension and the turbidity reading between galvanometer deflections of 10 and 65. Because of the large number of bacteria in suspension it was found necessary to dilute the contents of the culture tubes, usually from 10 to 40 cc., with 0.85 per cent saline solution in order to obtain valid measurements.

<sup>1</sup> Lumetron.



Transmitted light was measured in the same colorimeter which was also standardized for this procedure with barium sulfate suspension. A straight line was produced when the logarithm of the cc. of barium sulfate suspension was plotted against the optical density, the logarithm of the ratio of incident light to transmitted light ( $\log I_0/I$ ), between readings of 0.2 and 1.1 on the dial which was calibrated in the log ratio. Dilutions were also necessary for these measurements.

Cell volume was determined by use of Van Allen hematocrit tubes. The tubes were entirely filled with the thoroughly shaken bacterial suspensions and the volumes occupied by the cells were measured directly after centrifuging under standard conditions. The hematocrit tubes were calibrated for actual volume by weighing them first empty and then completely filled with water. The per cent cell volume was calculated from these data.

It is convenient in dealing with very wide dose ranges and also for purposes of biological assay to plot the response against the logarithm of the dose; consequently all results are so treated in Figs. 1 to 3 and 5.

### Results

*Effect of Rice Polishings Concentrate*—Since much greater amounts of acid were produced when large doses (quantity per test-tube) of rice polishings concentrate<sup>2</sup> were added to the medium of Pennington, Snell, and Williams than when amounts of pure calcium pantothenate alone (5) were added, it was obvious that the medium used could be improved. It was found that the treated yeast extract could be replaced with thiamine hydrochloride, nicotinic acid, and pyridoxine hydrochloride as suggested by Silber and Unna (8) with resulting increase in the slope of the calcium pantothenate curve. With the medium thus modified the acid production of *Lactobacillus casei* was studied with graded doses of calcium pantothenate and of rice polishings concentrate (Fig. 1). A range of dosage approximately 4000-fold was employed for both. A maximum of 9.5 to 10 cc. of 0.1 N acid produced in 72 hours was never exceeded when calcium pantothenate was used, whereas with rice polishings concentrate, after a similar plateau is reached at that level, increased dosage caused a sharp rise in acid production up to 22 cc. The curves in Fig. 1 indicated also that most of the acid production occurred during the first 48 hours and that the slopes of the initial steep linear portions of the standard calcium pantothenate curves were approximately equal to the slopes of the corresponding portions of the curves for the rice polishings concentrate.

The high acid production resulting when large amounts of the rice polishings concentrate were present is not to be explained on the basis of buffer-

<sup>2</sup> Ryzamin-B, Burroughs Wellcome and Company (U. S. A.), Inc.

ing action *per se*. Titrations of 10 cc. of medium containing the highest dose, 437 mg., used in this experiment showed that the buffering capacity was the same as that of the medium itself, since nearly equal quantities of 0.1 N HCl were required to titrate both solutions to pH 4. The above dose of the concentrate introduced less than 1 cc. of 0.1 N acid into the medium, an amount insignificant compared with the acid produced by the micro-organisms.

Curves of turbidity and of optical density (Fig. 2) resemble the acid production curve except that the plateaus are less distinct and shorter.

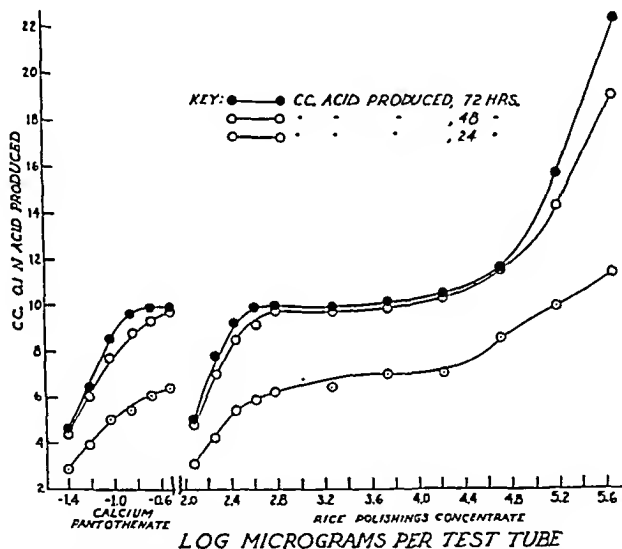


FIG. 1. Curves relating graded doses of calcium pantothenate and of rice polishings concentrate to cc. of 0.1 N acid produced during 24, 48, and 72 hour periods. Doses of calcium pantothenate up to 160  $\gamma$  have been used without any increase in titration above 10 cc.

This suggests that cell production may have continued when acid production has stopped in the middle dose range of the rice polishings concentrate. Added evidence that cell multiplication and acid production may not have been running parallel was supplied by the hematocrit studies (Fig. 2). Lack of correspondence between these two criteria along with the ability of the large doses of rice polishings concentrate to produce acid above the plateau level indicated that optimal conditions for bacterial metabolism were not present in the medium employed.

*Addition of Glucose*—Since the rice polishings concentrate contained

about 60 per cent of sugars and the medium contained only enough glucose to permit a production of about 11 cc. of 0.1 N lactic acid, it appeared that the carbohydrate might be the factor limiting acid production with large amounts of pure calcium pantothenate and with the intermediate plateau levels of the concentrate. Therefore, glucose was added to each of the lower doses of the rice polishings concentrate until the level of the carbohydrate (262 mg.) in the highest dose was reached. This was in addition to

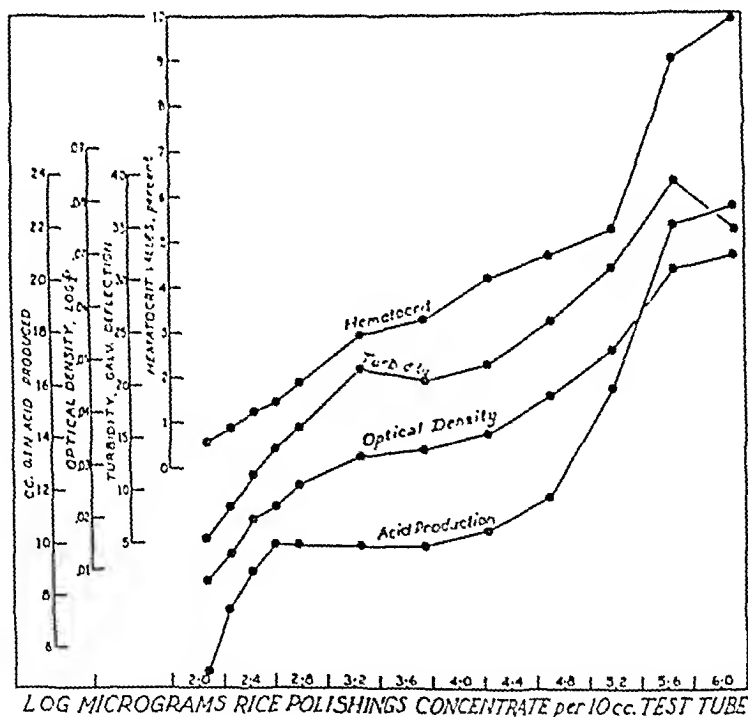


FIG. 2. Comparison of four methods of estimating the performance of *Lactobacillus casei* in 72 hours of incubation; graded doses of rice polishings concentrate plotted against acid production, transmitted light determinations (optical density), turbidity readings, and hematocrit values.

the amount already in the medium, 100 mg. per test-tube. A curve resulted with a shorter and more elevated plateau at the middle part of the dose range (Fig. 3). This indicated the possibility of still further improvement in the medium before the curve could become straightened out with removal of the central plateau. However, with the added glucose, cell development did parallel the acid production as indicated in Fig. 4, where the hematocrit values are plotted against the cc. of acid produced.

Doubling the glucose content of each tube in a series of dilutions of cal-

cium pantothenate did not increase the slope of the dose-response curve but did elevate the plateau level considerably. Thus, with 0.6  $\gamma$  of calcium pantothenate and 200 mg. of glueosc, 13 cc. of acid per tube were produced.

*Addition of Other Purified Substances*—Experiments with pure calcium pantothenate were continued. It was found that when a supplement containing several of the possible growth stimulants present in the rice polishings concentrate was added to the medium fortified with 362 mg. of glucose per test-tube, the plateau value was increased to 16 to 17 cc. of 0.1 N acid. This supplement contained the following in micrograms per test-tube: thiamine hydrochloride 100, pyridoxine hydrochloride 100,

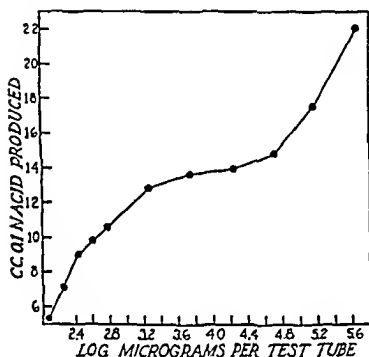


FIG. 3

FIG. 3. Curve relating graded doses of rice polishings concentrate to acid production, with glucose added to equal the amount present in the largest dose of the concentrate used.

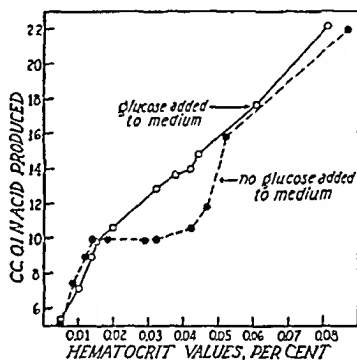


FIG. 4

FIG. 4. Curves relating hematocrit values to acid production, with and without the addition of glucose to the graded doses of rice polishings concentrate.

nicotinic acid 450, choline chloride 12,000, inositol 12,000, biotin<sup>3</sup> 2, and added riboflavin 100. However, this titration figure still falls short of that obtainable with the rice polishings concentrate.

When the medium of Landy and Dicken (2) became available, this was used with added glucose, 400 mg. per test-tube, so that insufficiency of substrate would not become the limiting factor in the production of acid by *Lactobacillus casei*. A few other modifications were also made (Medium A, Table I). A satisfactory dose-response curve for the calcium pantothenate series resulted (Fig. 5) with a steep linear portion ending on a high plateau for maximal acid production. Likewise, the curve for the rice polishings

<sup>3</sup> Concentrate of biotin, 100  $\gamma$  per cc., from the S. M. A. Corporation. This was the only substance used in the supplement which was not in pure form.

concentrate was improved by elimination of the plateau in the middle dose range. The improvement in this case seems to be due mainly to asparagine, for its omission reduces the maximum acid value from 20 to 15 cc. The traces of impurities known to be present in *c.p.* asparagine (9) may be responsible for some of the effect observed.

TABLE I  
*Ingredients of Modified Media*

The ingredients were diluted to 100 cc. after adjustment of the pH to 6.8. 5 cc. of this solution were subsequently diluted, in the assay, to 10 cc. by the addition of the solutions containing pantothenic acid.

	Medium A	Medium B	Medium C
Peptone preparation (3),* cc. . . . .		20	40
Casein hydrolysate (3), cc. . . . .		4	
"    "    (2), " . . . . .	20		
Na acetate, anhydrous, gm. . . . .	0.72		
Glucose, gm. . . . .	8	8	8
<i>l</i> -Asparagine, mg. . . . .	50	50	50
<i>l</i> -Tryptophane, mg. . . . .	20	20	20
<i>l</i> -Cystine, mg. . . . .	20	20	20
Salt Solution A (3), cc. . . . .	1	1	1
"    "    B (3), " . . . . .	1	1	1
Guanine HCl, mg. . . . .	1	1	1
Adenine SO <sub>4</sub> , " . . . . .	1	1	1
Xanthine, mg. . . . .	1	1	1
Uracil, mg. . . . .	1	1	1
Thiamine HCl, mg. . . . .	2	2	2
Riboflavin, mg. . . . .	2	2	2
Pyridoxine HCl, mg. . . . .	2	2	2
Nicotinic acid, " . . . . .	9	9	9
Choline chloride, " . . . . .	20	20	20
Inositol, mg. . . . .	20	20	20
Biotin (free acid), $\gamma$ . . . . .	8	8	
Folic acid,† $\gamma$ . . . . .	2.5	2.5	

\* The figures in parentheses represent bibliographic references.

† A folic acid concentrate, 5.5 per cent pure, was kindly furnished by Dr. R. J. Williams, Department of Chemistry, The University of Texas.

Equally satisfactory or slightly better curves of response with lower blanks and higher plateau values were obtained by using the purified ingredients of Medium A with the treated peptone and casein used by Pennington *et al.* (3) (see Medium B, Fig. 5 and Table I). It should be observed that with both of these media the higher doses of the rice polishings concentrate produced an upward trend from the plateau level of the calcium pantothenate curve, suggesting an additional stimulating factor.

However, since only the linear portion is used for assays and since the standard calcium pantothenate curve closely parallels the one for the concentrate, this factor may be disregarded when the concentrate is being assayed for pantothenic acid.

*Other Growth-Stimulating Factors*—That Medium B is capable of still further improvement is indicated by the fact that considerably higher titrations, between 35 and 40 cc. of 0.1 N acid, were produced when large amounts of yeast concentrates<sup>4</sup> or of liver extract<sup>5</sup> were tested with sufficient substrate. It is not known whether the extra stimulating factors are qualitatively or quantitatively different from those present in the basal medium. Pantothenic acid alone is apparently not this stimulating factor,

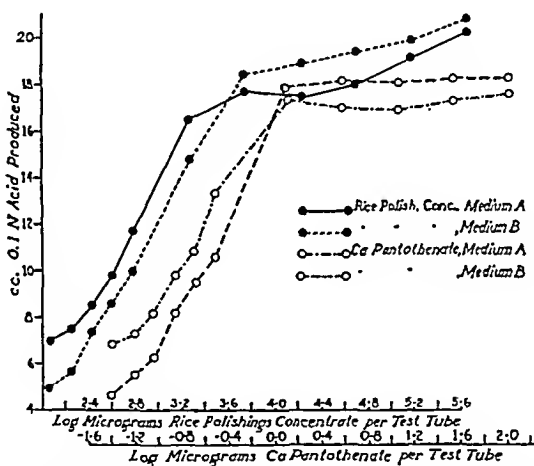


FIG. 5. Curves relating graded doses of rice polishings concentrate and calcium pantothenate to acid production. For Media A and B see Table I and the text.

because amounts of the pure substance much greater than that required for a 20 cc. titration value did not increase this maximum figure. It appears that biotin and folic acid may be ruled out also, for doubling the amounts of these present in Medium B, with maximum calcium pantothenate present, also resulted in titrations of 20 cc. The addition of glutamic acid to Medium B, suggested as a growth stimulant for *Lactobacillus casei* (10, 11), caused no greater response.

The omission of asparagine resulted in lower titrations, as would be

<sup>4</sup> Two yeast preparations were used: Bacto-yeast extract, Difco Laboratories, Inc., and a concentrate from the Yeast Products Corporation.

<sup>5</sup> Lilly.

expected (4, 10). However, biotin and folic acid may both be omitted, for their removal resulted in no change in the response to graded submaximal amounts of pure calcium pantothenate. It may be assumed, therefore, that the casein and peptone preparations provide enough biotin, folic acid, and glutamic acid for *Lactobacillus casei*.

Continued study of the media has shown that the casein hydrolysate may be omitted entirely if the peptone is doubled. With this modification (Medium C, Table I) the most satisfactory dose-response curve was obtained, which was linear between doses of 0.01 and 2.56  $\gamma$ . The resulting responses were from 4.5 to 22.5 cc. of 0.1 N acid. The range of dose formerly used with the unmodified medium employed previous to these studies was 0.04 to 0.14  $\gamma$ ; the responses were from about 4.5 to 9.5 cc. of 0.1 N acid. The results with increased peptone suggest that this material furnishes additional substances not heretofore provided in adequate amounts by the media, even to the extent of added buffer action (12).

When the photolyzed peptone preparation of Snell and Strong is substituted for the peptone in Medium C, and riboflavin is replaced by calcium pantothenate, preliminary studies have indicated that the medium thus modified is satisfactory for the assay of riboflavin. Curves of response show a similar relationship between the logarithm of the dose and titration values.

**Assay Procedure**—In the planning of pantothenic acid assays, doses are selected so that the responses will fall upon the linear portion of the dose-response curve. Doses of both the unknown and the standard are increased step-wise, each dose being 1.5 or 2 times the next lower dose. Three or more levels are used and each set up in triplicate, the titration method being employed to determine the response. For certain products, preliminary treatment is necessary in order to obtain maximum yields of pantothenic acid. For instance, predigestion of yeast extracts with malt diastase<sup>6</sup> increases the pantothenic acid potency considerably, as has been previously described (13).

From the data thus obtained, the potency may be determined conveniently and its accuracy evaluated by plotting all points on a semi-logarithmic dose-response curve and then employing graphic interpretations based on such statistical methods as those described by Bliss (14, 15). In assays of the above design, the slopes of the dose-effect curves have usually been between 9 and 11.

#### SUMMARY

1. An improved medium for use in microbiological assays for pantothenic acid by *Lactobacillus casei* has been described.

<sup>6</sup> Malt diastase (clarase), highly concentrated; Eimer and Amend.

2. The improvement in the medium has been effected by the omission of yeast extract, and the addition of extra glucose, asparagine, and other nutrients.

3. The improved medium allows a longer and steeper dose-response curve.

4. Evidence has been presented that the medium may be further improved to permit greater acid production when more information is obtained about growth-stimulating factors in natural products.

5. Use of the semilogarithmic dose-response curve for both unknown and standard is discussed.

The authors wish to express their thanks to Dr. Alfred H. Taylor for his cooperation in the use of the photoelectric colorimeter.

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# STUDIES IN MINERAL METABOLISM WITH THE AID OF ARTIFICIAL RADIOACTIVE ISOTOPES

## VII. THE DISTRIBUTION AND EXCRETION, PARTICULARLY BY WAY OF THE BILE, OF IRON, COBALT, AND MANGANESE

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The growing realization of the importance of the "trace" elements in the physiology of the animal organism makes it desirable to obtain more extensive information on the metabolism of these elements. Their minute requirements make it difficult to apply ordinary methods of chemical analysis for such investigations. In many instances this handicap can be overcome by means of tracer experiments with radioactive isotopes. Furthermore, the use of labeled atoms makes it possible to determine the fate of the dose administered of a given element.

Previous communications from this laboratory have dealt with certain aspects of the metabolism of iron (1), manganese (2), and cobalt (3). The present work was undertaken mainly to study the importance of the bile as a vehicle for the excretion of the above three elements into the intestinal tract. The isotope method is particularly well adapted for this purpose.

The rôle of the bile in the excretion of calcium and strontium in rats with bile fistulas was investigated and reported in a previous paper (4).

### *Methods*

For the experiments on bile, rats with an artificial gallbladder type of fistula, weighing between 400 and 500 gm., were used. The fistula was prepared according to the operation of Sawyer and Lepkovsky (5) as modified by Harrington, Greaves, and Schmidt (6). After the operation, the animals were given a saline-glucose solution (1 per cent NaCl, 10 per cent glucose). Rats allowed to drink this fluid voluntarily survived the operation for 1 to 2 weeks. The above rats were reared on the stock colony diet. To obtain information on the effect of a diet low in manganese on the partition of this element, certain experiments were carried out on rats without fistulas reared on a modified milk diet.<sup>1</sup>

The isotopes employed were Mn<sup>56</sup> with a half life of 310 days, Fe<sup>55</sup> with

<sup>1</sup> The composition of the modified milk diet used as the basal diet was powdered skim milk 75 gm., fat (Crisco) 15 gm., and sucrose 10 gm. The supplements were Fe 10 mg., Cu 0.5 mg., nicotinic acid 1.0 mg., and calcium pantothenate 2.0 mg.

a half life of 4 years, and a mixture of  $\text{Co}^{54, 55}$  with half lives of 72 and 270 days respectively (7). The methods of isolating the radioactive Mn and Co have been reported elsewhere (2, 3). The radioactive iron,  $\text{Fe}^{55}$ , was obtained by bombarding manganese with deuterons. The iron was precipitated four times from acid solution with cupferron, small amounts of inert Mn and Co being added each time as carriers. It was next precipitated four times with ammonium hydroxide, inert Zn being added as a carrier. It was then made up in neutral solution as ferric citrate.

Measurement of the radioactivity of the samples was made with a scale-of-eight circuit Geiger-Müller counter.<sup>2</sup> To detect the soft radiations of certain of the isotopes used, a bell-shaped counter tube with a thin mica window was employed.<sup>3</sup>

Determinations of  $\text{Mn}^{54}$  and  $\text{Co}^{54}$  were corrected for absorption of radiation by bile, tissue, or excreta ash. The  $\text{Fe}^{55}$  was first electroplated into tin capsules and read directly.

#### DISCUSSION

*Iron*—In experiments on dogs, Hahn, Whipple, and coworkers (8) found that the mammal shows only a slight ability to eliminate iron in any manner once it is incorporated into the body. Excretion in the bile reached significant proportions only under conditions of excessive blood corpuscle destruction and was small in animals of normal hematopoietic function. The results of our experiments on rats parallel the observations of Hahn, Whipple, and coworkers.

The  $\text{Fe}^{55}$  excreted in the bile is shown in Fig. 1, Curves 3 and 4. The amount in the bile was very little different upon oral or parenteral administration. In the case of parenterally administered  $\text{Fe}^{55}$ , the quantity appearing in the bile in 48 hours represented only 5 per cent of the amount in the gastrointestinal tract and feces. This is shown in Table I. The amount excreted into the urine was about 1.5 per cent of the administered dose in the case of both routes of administration. The excretion in the urine probably depends upon the degree of elevation attained by the plasma inorganic iron. Most of the excretion of  $\text{Fe}^{55}$  into the urine occurred in the first 30 hours after its administration.

Absorption of orally administered  $\text{Fe}^{55}$  was low even though the dose was small (0.1 mg.) and the bile fistula rats were in a fasting condition. This is shown by the high content of  $\text{Fe}^{55}$  found in the feces and gastrointestinal tract of animals given the  $\text{Fe}^{55}$  orally.

<sup>2</sup> Built by the Cyclotron Specialties Company, Moraga, California.

<sup>3</sup> A modification of a counter tube designed by Dr. Dodson of the California Institute of Technology (unpublished).

<sup>4</sup> The asterisk denotes that the elements were labeled with their respective radioactive isotopes.

True fecal excretion of iron appears to be of negligible proportion. The average figure of 1.9 per cent  $\text{Fe}^*$  found in the gastrointestinal tract of the injected rats supports the suggestion of Hahn, Whipple, and coworkers, that much of the fecal iron may be derived from the epithelial wastage of the stomach and intestine.

**Cobalt**—The literature on the metabolism of cobalt prior to the introduction of the isotope-labeling technique is scant, inconclusive, and contradictory.

The presence of cobalt in bile was demonstrated in 1884 by Stuart. This was substantiated by Caujolle but denied by Mascherpa (see (9)).

In tracer experiments with the radioactive element, Copp and Greenberg (3) observed that the urine is the chief path for the true excretion of

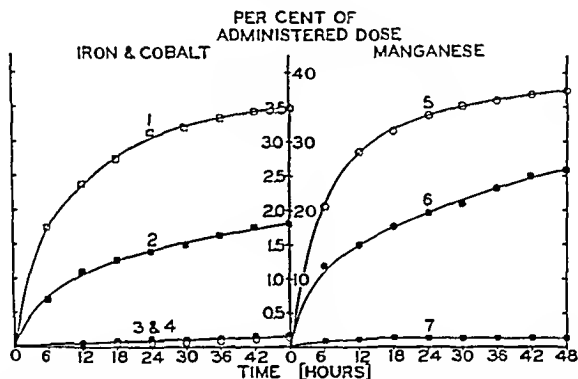


FIG. 1. The rate of elimination of  $\text{Fe}^*$ ,  $\text{Co}^*$ , and  $\text{Mn}^*$  in the bile. Curve 1,  $\text{Co}^*$  injected; Curve 2,  $\text{Co}^*$  orally; Curves 3 and 4,  $\text{Fe}^*$ ; Curve 5, 0.1 mg. of  $\text{Mn}^*$  injected; Curve 6, 0.01 mg. of  $\text{Mn}^*$  injected; Curve 7,  $\text{Mn}^*$  orally.

cobalt. This was corroborated by Kent and McCance (9) and the results of the present study. An average of 63.5 per cent of a 0.1 mg. dose of injected  $\text{Co}^*$  was eliminated by way of the urine and only 8.4 per cent appeared in the feces and bile (Table I). Upon oral administration, an average of 18.5 per cent appeared in the urine and about 40 per cent in the feces. In the latter case, the difference must be mainly due to incomplete absorption of orally administered cobalt. The absorbed cobalt is chiefly excreted by the kidneys. These results agree with the previous findings of Copp and Greenberg.

The course of the elimination of  $\text{Co}^*$  in the bile is shown in Curves 1 and 2 of Fig. 1. The bile is an important pathway for the passage of cobalt from the body into the intestinal tract. That it is not the sole path is shown by the data for feces in Table I. Of the injected  $\text{Co}^*$ , approxi-

mately equal quantities appeared in the bile and in the feces. As would be expected from the incomplete degree of absorption, only about half as

TABLE I  
*Partition of Labeled Iron, Cobalt, and Manganese, in Per Cent of Administered Dose*

	Mode of administration	Amount	No. of animals	Bile	Urine	Feces	Gastro-intestinal tract	Liver
Bile fistula rats								
Fe (48 hrs.)	Injection	0.1	3	0.1 ± 0.01	1.6 ± 0.5	0.2 ± 0.2	1.9 ± 1.5	
	Oral	0.1	2	0.2 ± 0.1	1.4 ± 0.9	28.5 ± 10.2	20.6 ± 3.3	
Co (72 hrs.)	Injection	0.1	5	3.5 ± 1.4	63.5 ± 8.5	4.9 ± 0.6		2.5 ± 0.6
	Oral	0.1	2	2.0 ± 0.2	18.5 ± 12.0	39.6 ± 12.0		3.5 ± 0.7
Mn (18 hrs.)	Injection	0.01	2	27.1 ± 0.4	5.4 ± 1.4	5.6 ± 0.9	21.2 ± 0.9	27.1 ± 3.5
	" "	0.1	3	37.3 ± 8.6	3.2 ± 2.1	6.5 ± 1.7	7.3 ± 1.0	11.7 ± 3.7
	Oral	0.1	2	1.1 ± 1.0	1.2	39.1 ± 2.3	42.7 ± 4.4	
Rats without fistulas on modified milk diet* (21 hrs.)								
Mn	Injection	0.01	4		0.9 ± 0.3	16 ± 8.0	39 ± 8.5	26.6 ± 1.9
	Oral	0.01	4		0.7 ± 0.1	44 ± 6.0	42 ± 2.0	1.4 ± 0.9
Rats without fistulas on stock colony diet								
Mn	Intravenously†	0.01	2		2.1 ± 0.3	30.3 ± 0.4	21.1 ± 0.8	11.8 ± 3.7
	" "‡	0.01	2		0.6 ± 0.3	49.2 ± 5.0	10.2 ± 0.9	17.0 ± 2.0

Administration of the element was by intraperitoneal injection, orally, or by intravenous injection. The figures given are mean values ± the mean deviation.

\* Two of the rats in each of these two groups were injected with 1 mg. of thiamine chloride every other day for four periods prior to the time of administering Mn\*.

† Analyses made after 24 hours.

‡ Analyses made after 48 hours.

much Co\* was found in the bile upon oral administration as upon parenteral administration.

The considerable amount of Co\* accumulating in the liver supports the suggestion that the bile plays an important part in the transport of cobalt

from the body to the intestine. The accumulation in the liver suggests that cobalt has a significant rôle in the functioning of the liver.

*Manganese*—The rôle of manganese in animal biology has been the subject of fairly extensive investigation. The literature on this topic, up to 1935, has been reviewed by von Oettingen (10). Interest in manganese has been heightened by the discovery that it is required for the prevention of perosis in birds (11), and its deficiency is a possible factor influencing the occurrence of lameness in pigs (12). In support of earlier observations, manganese deficiency has recently been shown to result in impaired growth and sterility in both male and female rats (13).

It appears to be quite well established that manganese is preferentially excreted into the alimentary canal and very little is eliminated by the way of the urine. Kobert in 1883 and Cahn in 1884 observed that large injected doses of manganese salts were mostly excreted with the feces and only traces appeared in the urine (see (10) for the references). Subsequent experimental work has supported this observation in the case of more physiological quantities. Greenberg and Campbell (2), using radioactively labeled Mn, found that 90.7 per cent of a 1 mg. dose injected intraperitoneally into a rat was eliminated with the feces.

Kent and McCance were unable to demonstrate any excretion of manganese injected intravenously in two of three human subjects. On the basis of this observation, they conclude that injecting small doses of manganese does not necessarily provoke an excretion of the metal. These authors criticize the work of Greenberg and Campbell because of the administration by intraperitoneal injection. It will be shown below that this does not alter the course of the excretion of manganese.

Manganese was detected in the bile of cats after its oral administration as early as 1860 by Wichert (10). Bargetto observed that the biliary excretion of manganese increased with the manganese content of the liver (14). Aside from these and a few other scattered observations, little information has appeared on the significance of the bile for the excretion of manganese.

The present experiments show that the bile, under the conditions studied, is the most important vehicle for the transport of manganese from the body into the intestine. The passage of  $Mn^{*}$  into the bile following its intraperitoneal injection is shown in Curves 5 and 6 of Fig. 1. When a 0.01 mg. dose was given, an average of 26 per cent accumulated in the bile and with a 0.1 mg. dose an even greater fraction (37.3 per cent) appeared in this fluid in 48 hours. The  $Mn^{*}$  in the bile under these conditions was from 1 to 3 times the quantity found in the alimentary tract and feces. Consequently, from 50 to 75 per cent of the injected manganese appearing in the feces is probably carried by the bile.

The kidneys play a very minor rôle in the excretion of  $Mn^{*}$  even when it

is injected. At most, the amount excreted into the urine is only a small fraction of the total dose.

When the  $Mn^*$  was given orally, only about 1 per cent was found in the bile (Fig. 1, Curve 6). Since 25 to 35 per cent of an injected dose of  $Mn^*$  is excreted in the bile, this indicates that only 3 to 4 per cent of  $Mn^*$  was actually absorbed from the intestine. The small amount of  $Mn^*$  in the liver confirms this. It is probable that this poor absorption of Mn may be an important factor in producing Mn deficiency.

The livers of the injected animals accumulated a large fraction of the  $Mn^*$ . This would appear to indicate that manganese in excess of the tissue needs is transmitted to the liver preliminary to its excretion.

A moderate degree of manganese deficiency changes the picture of manganese metabolism but little. This is shown by the  $Mn^*$  partition in the rats reared on the modified milk diet. This differs but little from the picture observed in the bile fistula animals.

The livers of the milk-fed rats were low in manganese, the average being  $0.085 \pm 0.008$  mg. per 100 gm. of fresh weight, in contrast to normal values of about 0.25 mg. per 100 gm.

The objection raised by Kent and McCance to intraperitoneal injection was shown to be groundless by the experiments on stock colony rats without fistulas (Table I). Four young adult rats weighing 200 gm. were lightly anesthetized with ether, and injected with 0.1 mg. of  $Mn^*$  (as  $MnCl_2$ ) in 0.2 ml. of physiological saline, through the femoral vein. The animals were then placed in individual metabolism cages and the excreta collected in the usual manner. Two of the rats were sacrificed at 24 hours and the other two at 48 hours. The results of the experiments show that, when the  $Mn^*$  is given intravenously, a large fraction is quickly excreted into the intestinal tract. In 24 hours the feces and digestive tract contained around 51.5 per cent of the dose given, and in 48 hours, about 59.5 per cent of the dose. The liver showed the same marked degree of accumulation of  $Mn^*$  as was found upon intraperitoneal injection. The size of the dose injected cannot explain the difference between the present experiments and those of Kent and McCance. In the rat the manganese injected was between 0.05 and 0.5 mg. per kilo of body weight. The subjects used by Kent and McCance received 0.3 to 0.5 mg. of Mn per kilo.

Some manganese is transmitted from the blood into the milk. A litter of three rats was born to a female rat maintained on the milk diet. The rat was injected with 0.05 mg. of  $Mn^*$  intraperitoneally 2 days after parturition. Two of the young were sacrificed 3 days after the injection and the  $Mn^*$  determined. The bodies of the two rats contained 0.3 per cent of the dose. The remaining young was sacrificed 10 days following the injection. Its body contained 1 per cent of the  $Mn^*$  given the mother.

We are indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for the radioactive isotopes used in these experiments. We are grateful to Miss Frances M. Troescher for assisting us with a number of the experiments.

#### SUMMARY

1. The radioactive isotopes of iron, cobalt, and manganese were used to study the partition and mode of excretion of these elements. The importance of the bile as a vehicle for the excretion of the above elements was tested by employing an artificial gallbladder type of fistula.

2. Parenterally administered iron is excreted to only a slight degree, and is largely retained in the body. Only a trace of labeled iron appeared in the bile and but little more in the fecal contents.

3. The urine is the chief pathway for the excretion of cobalt. Orally administered cobalt is only partially absorbed and thus a large proportion passes through the intestinal tract and is eliminated with the feces. The bile is an important but is not the sole pathway for the passage of cobalt from the body into the intestinal tract. Considerable amounts of labeled cobalt accumulate in the liver.

4. Manganese is excreted almost totally with the feces and very little is eliminated in the urine. The bile plays a rôle of great importance in the intestinal excretion of manganese. Probably 50 to 75 per cent of the manganese that makes its way from the body into the intestinal canal is carried by the bile.

Orally administered manganese is very poorly absorbed even if the animal is in a fasting condition. The liver accumulated 10 to 30 per cent of parenterally administered, labeled manganese. Manganese in excess of the tissue needs may be transmitted to the liver preliminary to its excretion.

Manganese injected into the body of a lactating female is transmitted into the milk in small quantities.

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# RADIOACTIVE IODINE AS AN INDICATOR OF THE METABOLISM OF IODINE

## VI. THE FORMATION OF THYROXINE AND DIIODOTYROSINE BY THE COMPLETELY THYROIDECTOMIZED ANIMAL\*

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The early discovery that physiological activity in the thyroid gland was associated with an iodoprotein (1, 2) led logically to investigations of the physiological effects and chemical properties of various proteins or protein fractions that had been subjected to artificial iodination. In 1933-34, Abelin *et al.* observed physiological effects resembling those produced by the thyroid gland after the administration of fractions obtained by hydrolysis of iodinated protein (3, 4). It was not until 1939, however, that Ludwig and von Mutzenbecher (5) clearly demonstrated the isolation of crystalline thyroxine from the following proteins after treatment with molecular iodine: casein, serum albumin, serum globulin, silk fibroin, and edestin. The chemical identity of thyroxine was established by elementary analysis, determination of physical and chemical properties, and by the preparation of thyronine by catalytic hydrogenation of the thyroxine. These workers also obtained diiodotyrosine and monoiodotyrosine from these iodinated proteins. The isolation of crystalline diiodotyrosine, however, from a hydrolysate of iodocasein had been reported earlier (1910) by Oswald (6), whereas the preparation of thyroxine from iodinated casein was later (1939) confirmed by Harington and Rivers (7). Interestingly enough, von Mutzenbecher (8) has further shown that thyroxine is formed when diiodotyrosine is incubated with NaOH for 14 days, an observation that has received recent confirmation from Block (9) and Johnson and Tewkesbury (10). The latter (10) have also shown that pyruvic acid is a product of this reaction and on the basis of this finding have proposed a possible mechanism to explain the formation of thyroxine.

Since the early work of Abelin, physiological effects closely resembling thyroid activity have repeatedly been observed to follow the administration of iodinated protein and fractions of their hydrolysates (11-14). But

\* Aided by grants from the Commonwealth Fund and the Committee for Research in Endocrinology of the National Research Council.

the chemical significance of these findings is not clear at the present time, since in no case have they been correlated quantitatively with a definite organic molecule in the mixture fed.

The observations recorded above, particularly those dealing with the actual identification of thyroxine and diiodotyrosine in the hydrolysates of iodinated proteins, raised the interesting question whether thyroxine and diiodotyrosine can be formed elsewhere in the body than in the thyroid gland.<sup>1</sup> The demonstration of the presence of thyroxine and diiodotyrosine in the *thyroidless* animal does not necessarily prove that tissues other than the thyroid can synthesize these compounds; they could have been synthesized before excision of the thyroid glands and stored in the tissues. This particular objection can be overcome with the use of a tagged iodine. In the present investigation the ability of the completely thyroidectomized animal to form diiodotyrosine and thyroxine was tested with the aid of a labeled iodine. Radioactive iodine was administered several weeks after excision of the thyroid gland; radiodiiodotyrosine and radiothyroxine, if found thereafter, must have been formed only after excision of the thyroid glands. The completeness of thyroidectomy was established by two procedures, (1) histological examination of all serial sections of tissues of the neck and thorax, and (2) a procedure involving the iodine-concentrating capacity of thyroid tissue.

## EXPERIMENTAL

### *Thyroidectomized Rats*

The rats shown in Table I were thyroidectomized at the age of 4 to 6 months. For 2 to 8 months thereafter they were maintained on a stock diet. Each rat then received intraperitoneally 1 cc. of an isotonic NaCl solution containing tracer amounts of radioiodide ( $1 \times 10^7$  counts per minute). The preparation of tracer amounts of  $I^{131}$  has been described elsewhere (16, 17).

At intervals of 2 to 96 hours after the injection, the animals were anesthetized with nembutal. Blood was then removed by heart puncture and centrifuged. 3 cc. samples of plasma were pooled from each of two rats, thus making 6 cc. available for each analysis. The small intestine was removed, washed through with isotonic saline, weighed, and minced, and

<sup>1</sup> Chapman has suggested the conversion of iodine to a thyroxine-like substance in tissues other than the thyroid (15). This suggestion was based in part on the observation that the oxygen consumption was higher in thyroidectomized rats maintained on a high iodine diet than in those kept on a low iodine diet. It is questionable whether small increases in oxygen consumption in thyroidectomized rats can be accepted as evidence for the formation of a thyroxine-like substance. Furthermore, the absence of thyroid tissue in Chapman's thyroidectomized rats was not established.

samples weighing approximately 5 gm. were taken for analysis. Liver and gastrocnemius muscle were similarly weighed, chopped, and sampled. In some cases somewhat less than 5 gm. of muscle was available from both gastrocnemii; in such cases a small amount of muscle was removed from the thigh to make up the required amount. The plasma and tissues were

TABLE I  
*Formation of Thyroxine and Diiodotyrosine by Thyroidectomized Rats*

Rat No.	Body weight	Interval after thyroidectomy	Oxygen consumption		Tissue	Time after injection of I <sup>131</sup>	Administered I <sup>131</sup> recovered per gm.	I <sup>131</sup> of tissues found as		
			Per sq.m. per 24 hrs.	Decrease below normal*				Thyroxine	Diiodotyrosine	Inorganic
	gm.	mos.	liters	per cent		hrs.	per cent	per cent	per cent	per cent
131	395	8	97	47	Small intestine	2	0.132	1.5	13.6	87.4
132	385	2	96	48	" "	2	0.143	1.5	9.4	90.0
131					Liver	2	0.0733	1.9	12.6	88.4
132					"	2	0.0648	1.7	11.4	85.1
134	290	4	92	50	Small intestine	24	0.110	1.9	20.1	78.4
137	310	8	108	41	" "	24	0.0494	3.9	19.9	69.6
134					Liver	24	0.0692	1.8	20.5	77.5
137					"	24	0.0444	4.5	15.2	80.4
134					Muscle	24	0.0408	1.3	8.1	88.0
137					"	24	0.0190	3.3	12.0	83.1
134†										
135†	285	2	109	41	Plasma	24	0.212	1.0	7.01	91.8
136†										
137†	415	8	101	45	"	24	0.126	0.8	12.2	86.9
140	375	2	95	48	Small intestine	96	0.00583	8.0	13.0	74.7
141	415	2	105	43	" "	96	0.00861	7.2	15.0	77.4
140					Liver	96	0.00563	5.8	21.2	73.7
141					"	96	0.00957	8.6	21.3	69.9

\* The mean value observed by one of us (E. Anderson) for normal fed rats maintained under conditions identical with those of the thyroidectomized rats recorded in this study was  $184 \pm 3$  liters per sq.m. per 24 hours.

† The plasma was pooled from Rats 134 and 135.

‡ The plasma was pooled from Rats 136 and 137.

hydrolyzed and analyzed for radiothyroxine, radiodiiodotyrosine, and radioiodide by the method already described (17-19).

Determinations of oxygen consumption for the rats recorded in Table I were made a few days before the injection of the radioiodine. The Benedict multiple chamber apparatus (20) was used to determine the oxygen consumption of rats. The animals were kept in a constant temperature room at  $28.5^\circ$  for 24 hours before the measurements were made.

Since hypophysectomized rats (see below) do not tolerate fasting, all measurements of oxygen consumption were made during the absorptive state. The values, expressed as liters per sq.m. of body surface per 24 hours, were 41 to 50 per cent below the mean value observed by one of us (E. A.) for normal fed rats (Table I).

The distribution of the administered  $I^{131}$  among thyroxine, diiodotyrosine, and iodide fractions is shown in the last three columns of Table I. At the 24 hour interval after the injection, the radioiodine in these three fractions was measured in the small intestine, liver, muscle, and plasma, whereas at 2 and 96 hour intervals measurements were made in only the small intestine and liver.

*Diiodotyrosine*—A rapid conversion of the administered radioiodide to diiodotyrosine is shown in Table I. By as early as 2 hours, each gm. of small intestine contained about 0.1 per cent of the injected radioiodine, and as much as 14 per cent of this  $I^{131}$  was present as diiodotyrosine. At this same time interval as much as 13 per cent of the liver radioiodine was present as diiodotyrosine.

At the 24 and 96 hour intervals somewhat more (15 to 21 per cent)  $I^{131}$  of the small intestine and liver was present as diiodotyrosine. Somewhat smaller amounts of the  $I^{131}$  were found as diiodotyrosine in muscle and plasma.

From the results as a whole it appears that radiodiiodotyrosine attains a maximum value very soon.

*Thyroxine*—Less than 2 per cent of the  $I^{131}$  contained in intestine and liver was found in the form of thyroxine at the 2 hour interval. Although these low values are of slight significance, the larger amounts found at 24 hours and, more particularly, at 96 hours in small intestine are evidence that the rats recorded in Table I formed thyroxine. Thus at 96 hours 8 per cent of the  $I^{131}$  of the small intestine and as much as 8.6 per cent of that of the liver were incorporated into thyroxine.

*Inorganic*—The largest fraction of  $I^{131}$  of each tissue was present in the inorganic form at all three intervals examined.

#### *Thyroidectomized-Hypophysectomized Rats*

Accessory thyroid tissue, if present, or fragments of the gland missed at operation, would be depressed by the additional removal of the hypophysis. For this reason rats were subjected to both thyroidectomy and hypophysectomy. The rats shown in Table II were thyroidectomized first and hypophysectomized 9 days later. 7 weeks after hypophysectomy each animal was injected intraperitoneally with 2 cc. of an isotonic NaCl solution containing a tracer dose of radioiodide. All four rats were anesthetized with nembutal 96 hours after the administration of the radioiodine.

Blood was removed by heart puncture and centrifuged for the separation of plasma. Liver and small intestine were then excised and treated in the manner described above. Determinations of the oxygen consumption of the rats recorded in Table II were made on several occasions after hypophysectomy, the last determination coming 24 hours before they were sacrificed.

No difference was observed between the thyroidectomized rat (Table I) and the rat subjected to both thyroidectomy and hypophysectomy as regards the distribution of  $I^{131}$  of the small intestine and liver among diiodotyrosine, iodide, and thyroxine. Thus 20 to 24 per cent of the  $I^{131}$  contained in either the small intestine or in the liver of the thyroidec-

TABLE II  
*Formation of Thyroxine and Diiodotyrosine by Thyroidectomized-Hypophysectomized Rats*

Rat No.*	Body weight	Oxygen consumption		Tissue	$I^{131}$ recovered per gm. 96 hrs. after injection	$I^{131}$ of tissues found as		
		Per sq.m. for 24 hrs.	Decrease below normal			Thyroxine	Diiodotyrosine	Inorganic
	gm.	liters	per cent		per cent	per cent	per cent	per cent
142	204	94	49	Liver	0.0155	3.9	24.1	72.0
142	204			Small intestine	0.0165	3.7	20.3	76.1
143	200	115	38	Liver	0.0151	5.4	20.6	67.7
143	200			Small intestine	0.0111	3.8	19.6	75.0
144	203	112	39	Liver	0.0126	4.6	23.7	72.0
144	203			Small intestine	0.00804	7.8	21.7	70.7
145	190	104	43	Liver	0.00945	4.9	18.0	74.0
145	190			Small intestine	0.0106	8.6	21.5	68.4

\* All rats were 65 days old at the time of thyroidectomy. 9 days after thyroidectomy they were hypophysectomized. An interval of 7 weeks followed before the experiment was carried out.

tomized-hypophysectomized animals was found as diiodotyrosine. As much as 8.6 per cent of the  $I^{131}$  of the small intestine was present as thyroxine. Approximately 25 to 30 per cent of the  $I^{131}$  of these tissues was organically bound.

*Formation of Thyroxine and Diiodotyrosine in Rats Shown to Be Completely Thyroidectomized by Radioautographic and Histological Methods*

Proof as to whether thyroxine and diiodotyrosine can be formed in the absence of all thyroid tissue can only be as valid as the evidence provided for completeness of thyroidectomy. The procedures commonly employed to establish completeness of thyroidectomy are as follows: (1) gross in-

spection of the site of operation. This examination, even when done with the binocular microscope, may fail to identify minute fragments of thyroid tissue; (2) examination of the operative region by means of serial sections. To exclude the presence of all thyroid tissue, however, serial sections must be made of the entire path of development of the thyroid gland. This involves all structures from the base of the tongue to the heart and, besides the tongue, heart, and pericardium, includes the esophagus, larynx, trachea, thymus, and related structures. The preparation and examination of serial sections of such an amount of tissue are tedious and time-consuming processes. Even a careful observer, moreover, may fail to detect small isolated groups of cells of thyroid epithelium in the absence of typical follicular morphology; (3) measurements of oxygen consumption or other metabolic tests can only be regarded as presumptive indications of the completeness of thyroidectomy. A previous study (21) has shown that a low oxygen consumption in a thyroidectomized rat is not incompatible with the presence of extremely small fragments of iodine-concentrating tissue, presumably thyroid.

To test the completeness of thyroidectomy in the present study use was made of the known ability of thyroid tissue to concentrate iodine, and in addition histological examination was made of *all* serial sections of *all* tissues in the neck and chest regions.

Rats were thyroidectomized at the age of 38 to 41 days. 13 days later each rat received intraperitoneally 1.7 cc. of an isotonic NaCl solution containing tracer amounts of radioiodine ( $1 \times 10^7$  counts). The animals were anesthetized with nembutal 114 hours after the injection of the  $I^{131}$ . Plasma, liver, and small intestine were removed for determination of their radiothyroxine, radiiodiiodotyrosine, and inorganic radioiodine contents.

The animals were then subjected to the following examination to determine the completeness of thyroidectomy. The site of operation was examined under a binocular microscope. Radioautographs (21) were then made from two tissue masses. The region normally occupied by the thyroid gland was removed *in toto*; this included trachea, larynx, and esophagus. The second tissue mass included all tissues in which one might expect to find either aberrant or operatively misplaced thyroid tissue; namely, the base of the tongue, the remainder of the trachea and esophagus, thymus, heart and pericardium, neck muscles, and great vessels. The two tissue masses were placed on a filter paper, covered with a thin sheet of cellophane, and rolled flat. The above preparations were then placed in an x-ray cassette. A sensitive "non-screen" x-ray film was placed in contact with the cellophane and the cassette closed. Exposures were made over varying intervals (1, 19, and 48 hours).

The results obtained on three thyroidectomized rats are recorded in

Table III. In Rats 2 and 9, there was a complete absence of all thyroid tissue as shown by (1) gross inspection of the thyroid region, (2) failure to demonstrate iodine-concentrating tissue in the neck and chest regions by radioautographic methods,<sup>2</sup> and (3) failure to detect thyroid tissue after histological examination of *all* serial sections of the entire neck and chest regions.

A fragment of iodine-concentrating tissue was left in the region of the thyroid gland of Rat 3. Observations on this rat were included in Table III to provide a comparison with Rats 2 and 9.

TABLE III

*Formation of Thyroxine and Diiodotyrosine in Rats Tested for Completeness of Removal of Thyroid Tissue*

The animals were killed 114 hours after the injection of  $I^{131}$ .

Rat No.	Weight	Test for completeness of thyroidectomy			Tissue	Administered <sup>131</sup> I recovered per gm.	<sup>131</sup> I of tissues recovered as		
		Histology*	Radioautographs*				Thyroxine	Diiodotyrosine	Inorganic
			Thyroid region	Neck and chest region					
	gm.					per cent	per cent	per cent	per cent
2	130	Complete	Complete	Complete	Plasma	0.00412	5.4	8.0	86.5
2					Small intestine	0.00265	5.2	17.9	76.9
2					Liver	0.00190	8.8	33.2	58.0
9	126	"	"	"	Plasma	0.00469	4.1	17.0	78.9
9					Small intestine	0.00153	13.6	22.8	63.6
9					Liver	0.00118	11.5	31.1	57.4
3	126	Frag- ment†	Incom- plete	"	Plasma	0.0182	16.0	13.9	70.1

\* See the text for a description of the procedure.

† This fragment was left in the normal location of the thyroid gland and was less than 1.0 mm. in diameter.

Newly formed thyroxine and diiodotyrosine were found in the tissues of rats (Nos. 2 and 9) shown to be deprived of all thyroid tissue. As much as 14 per cent of the  $I^{131}$  of the small intestine was present as thyroxine 114 hours after the injection of the labeled iodine. There can be no question of the ability of either Rat 2 or Rat 9 to convert iodide to diiodotyrosine. The largest conversion was observed in the former animal; 33 per cent of its liver  $I^{131}$  was present as diiodotyrosine.

<sup>2</sup> It is of interest to note that four of a series of twelve thyroidectomized rats showed iodine-concentrating tissue (presumably thyroid tissue) in the mediastinal region, by the above radioautographic procedures. This means that these animals could not have been completely thyroidectomized by the usual surgical technique.



The plasma of Rat 3, in which a fragment of thyroid tissue remained, contained more than 3 times as much of the administered  $I^{131}$  as did the plasma of Rats 2 and 9. Moreover, the fraction of the  $I^{131}$  of the plasma found in the form of thyroxine was much greater in Rat 3 than in Rat 2 or 9.

*Identification of Radiothyroxine and Radiodiiodotyrosine Isolated from Plasma of Thyroidectomized Rats by Their Recrystallization to Constant Specific Activity*

The possible occurrence of iodinated compounds other than thyroxine and diiodotyrosine in animal tissues should be considered. Although diiodothyronine has not been found in animal tissues, it would, if present, appear mainly in the diiodotyrosine fraction in the analysis used in the present investigation (22). Monoiodotyrosine has recently been isolated by Ludwig and von Mutzenbecher (5) from iodinated casein and by Herrriott from iodinated pepsin (23); if present in animal tissues, this substance would probably follow the diiodotyrosine fraction. The procedure by which the actual presence of radiothyroxine and radiodiiodotyrosine in the plasma of the thyroidectomized rat was established is similar in principle to one described elsewhere (24).

1.5 cc. of plasma were pooled from each of the four rats listed in Table II. The 6 cc. of plasma so obtained were hydrolyzed with 60 cc. of 2 N NaOH for 8 hours on the steam bath. The resultant hydrolysate was cooled and acidified carefully with concentrated HCl. A total of 4 cc. of 0.02 M KI was added as carrier. After oxidation with  $KIO_3$  the inorganic radioiodine was extracted six times with  $CCl_4$ . The aqueous layer containing the organically bound radioiodine was adjusted to pH 3.5 to 4.0 with 2 N NaOH. Two extractions with butyl alcohol were then made to remove thyroxine, the first with 50 cc. and the second with 25 cc. The butyl alcohol fractions were combined and reextracted with two portions (75 and 35 cc.) of 20 per cent technical NaOH to remove the diiodotyrosine. These two alkaline washings were combined with the initial aqueous layer. The butyl alcohol layer was evaporated to dryness under reduced pressure. 50 mg. of non-radioactive crystalline thyroxine were added to the butyl alcohol residue as carrier and the mixture taken up in hot 0.10 N  $K_2CO_3$ . The thyroxine was recrystallized by way of the K salt, as described by von Mutzenbecher (8).

The alkaline aqueous fraction containing diiodotyrosine was evaporated to about 30 cc. under reduced pressure and acidified to pH 3.5 to 4.0 with HCl. Six extractions with 30 cc. portions of butyl alcohol were made. These butyl alcohol layers, which now contained the diiodotyrosine, were combined and evaporated to dryness under reduced pressure. 50 mg.

of non-radioactive crystalline diiodotyrosine were added to the butyl alcohol residue and the mixture taken up in hot 70 per cent ethyl alcohol.

The thyroxine and diiodotyrosine were each recrystallized five times further, the thyroxine as the potassium salt and the diiodotyrosine from 70 per cent ethyl alcohol. In each recrystallization of thyroxine, 10 mg. of non-radioactive diiodotyrosine were added in order to *wash out* any contaminating radiodiiodotyrosine by dilution. In the case of the diiodotyrosine recrystallization, 10 mg. of non-radioactive thyroxine were added each time for the same purpose. After each recrystallization the specific activities of the thyroxine and diiodotyrosine were determined according to the procedure described elsewhere (24).

Table IV shows that a constant amount of radioactivity remained associated with each compound despite repeated recrystallizations and washings. It should be observed that the specific activities of both thyroxine

TABLE IV

*Recrystallization of Thyroxine and Diiodotyrosine to Constant Specific Activity*

The specific activity is measured by the ratio of the radioactivity to the colorimeter reading.

Recrystallization	Thyroxine			Diiodotyrosine		
	Colorimeter reading	Radioactivity <i>counts per min.</i>	Specific activity	Colorimeter reading	Radioactivity <i>counts per min.</i>	Specific activity
1st	542	285	0.53	322	595	1.85
2nd	470	220	0.47	408	690	1.69
3rd	630	270	0.43	394	635	1.61
4th	534	250	0.47	330	520	1.58
5th	555	240	0.43	368	610	1.66

and diiodotyrosine did not change significantly after the second recrystallization. This would be expected to occur only in a case in which the substance giving the radioactivity was identical with the material that underwent repeated recrystallization. If two substances were involved, one being adsorbed on the other, it is not likely that a constant specific activity would be obtained by repeated recrystallizations and washings. It was shown elsewhere that, when radioiodide or radiodiiodotyrosine was added to non-radioactive crystalline thyroxine, only two recrystallizations of the latter were necessary to remove the radioactive substances completely from the thyroxine. This was also found to be true when radioiodide or radiothyroxine was added to non-radioactive diiodotyrosine and recrystallizations of the latter carried out. No radioactivity was found associated with the crystalline diiodotyrosine after its second recrystallization (24).

The results indicate the presence of newly formed thyroxine and diiodotyrosine in the thyroidectomized-hypophysectomized animal; the  $I^{131}$  in the thyroxine-like fraction cannot be due to contamination with radio-diiodotyrosine or radioiodide, and the  $I^{131}$  in the diiodotyrosine-like fraction cannot be ascribed to contamination with radiothyroxine or radioiodide.

*Reliability of Quantitative Procedures Employed for Measurement of Thyroxine and Diiodotyrosine*

In view of the low value of  $I^{131}$  of the tissues found as thyroxine, it is of interest to note here the validity of the quantitative procedure employed for the determination of the three iodine fractions. The following experi-

TABLE V  
*Recovery of Added Thyroxine and Diiodotyrosine*

Experiment No.	Radioactivity initially present as	Initial radioactivity recovered as		
		Thyroxine	Diiodotyrosine	Inorganic iodine
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Iodide	0.61	0.83	98.6
	"	0.17	0.28	99.5
	"	0.82	0.60	98.5
	"	0.69	0.81	98.8
2a	<i>l</i> -Diiodotyrosine	0.70	96.8	3.0
	"	1.0	97.4	1.8
2b	<i>dl</i> -Diiodotyrosine	0.95	98.0	0.01
	"	0.69	97.9	0.99
3a	<i>dl</i> -Thyroxine	96.6	3.4	0.01
	"	98.1	1.8	0.00
3b	"	87.2	5.82	6.95
	"	88.3	5.40	6.30

ments, in addition to demonstrating the accuracy with which radiothyroxine, radiodiiodotyrosine, and radioiodide can be measured in tissues, show that owing to a slight loss the thyroxine values recorded in Tables I to III are slightly low.

*Experiment 1*—Radioiodide was added to non-radioactive desiccated thyroid tissue and carried through the hydrolysis and fractionation procedure.

*Experiment 2*—Radiodiiodotyrosine was prepared in two ways, (a) by synthesis according to Harington's method (25) and (b) by isolation from the thyroid gland of a rat injected with radioiodine, as described above in the section on recrystallization. The radiodiiodotyrosine so isolated was added to non-radioactive desiccated thyroid tissue and the latter hydrolyzed and fractionated in the usual manner.

*Experiment 3*—Radiothyroxine was isolated from the thyroid of a rat previously injected with radioiodine in the manner described above in the section on recrystallization. The radiothyroxine so obtained was treated in two ways to determine the effect of the hydrolysis procedure upon thyroxine recovery: (a) radiothyroxine was added to non-radioactive desiccated thyroid tissue previously hydrolyzed with 2 N NaOH and this mixture was then extracted with butyl alcohol according to the usual procedure for fractionation; (b) radiothyroxine was added to non-radioactive desiccated thyroid tissue and the whole mixture subjected to hydrolysis and fractionation in the usual manner.

The results of the above experiments are given in Table V. A slight breakdown of diiodotyrosine occurs in the procedure. Approximately 12 per cent of the thyroxine is decomposed during the hydrolysis; the radioiodine from the decomposed radiothyroxine appears equally distributed between the diiodotyrosine-like and inorganic iodine fractions. It is thus evident that the values for the thyroxine-like fractions shown in Tables I to III are probably low.

#### DISCUSSION

The results of the present investigation show that rats whose metabolic rate has been reduced considerably by excision of the thyroid gland or of both thyroid and pituitary glands are still able to convert inorganic iodine to diiodotyrosine. Inert crystalline diiodotyrosine was added to the radioactive diiodotyrosine-like fraction extracted from plasma; repeated recrystallizations of diiodotyrosine so labeled yielded a constant radioactivity per unit amount of crystalline diiodotyrosine. This observation would leave little doubt that the injected iodide was converted into diiodotyrosine by the tissues of the thyroidectomized rat. Indeed, as early as 24 and 96 hours after the introduction of radioiodine, 20 per cent of the radioiodine contained in the liver and in the small intestine was found organically bound as diiodotyrosine. A comparison between the results obtained in the present investigation and those previously obtained in the normal animal (17) suggests that the rate at which injected inorganic radioiodine appears as diiodotyrosine in the liver and small intestine is not diminished by excision of the thyroid glands, although it should be pointed out that values cannot be assigned to the absolute amounts taking part in this conversion. The formation of diiodotyrosine was also shown to occur in rats in which the absence of all thyroid tissue was demonstrated.

The conversion of inorganic iodine to thyroxine is shown here to occur in the rat deprived of its thyroid glands for several months. The presence of newly formed thyroxine was definitely established by demonstrating a constant specific activity in the thyroxine obtained by five separate recrystallizations from a mixture of inert crystalline thyroxine which had

been added to the thyroxine-like fraction removed from the tissues. 96 hours after the injection of inorganic radioiodine, as much as 10 per cent of the radioiodine contained in the liver and small intestine was present as thyroxine. These amounts of radiothyroxine cannot be explained by experimental errors in the extraction procedures. The conversion of iodide to thyroxine was also observed in rats in which the absence of all thyroid tissue was demonstrated experimentally.

The question arises what tissues are involved in the formation of thyroxine and diiodotyrosine in the rat deprived of all thyroid tissue. A comparison of the specific activities of thyroxine and of diiodotyrosine in plasma and tissues would yield interesting information on this point. Thus higher specific activities of thyroxine and diiodotyrosine in muscle, liver, or small intestine than in plasma would show that plasma is not contributing appreciable amounts of these compounds to these tissues. To make this conclusion valid, specific activities should be measured at a time before their maximum values are attained. Such studies are in progress in this laboratory.

#### SUMMARY

1. The conversion of iodide to diiodotyrosine and thyroxine was measured in rats whose oxygen consumptions had been reduced considerably by excision of all visible thyroid tissue. As early as 96 hours after its injection, 30 per cent of radioiodine contained in the liver and in the small intestine was organically bound, 20 per cent as diiodotyrosine and as much as 8 per cent as thyroxine.

2. The presence of newly formed radiodiiodotyrosine and radiothyroxine was established in rats that had been deprived of their thyroid glands for several months by demonstrating a constant radioactivity per unit of crystalline material obtained during several recrystallizations of (1) a mixture of the thyroxine-like fraction of the tissues and inert crystalline thyroxine and (2) a mixture of the diiodotyrosine-like fraction of the tissues and inert crystalline diiodotyrosine.

3. The conversion of iodide to thyroxine and diiodotyrosine was demonstrated in rats shown to be completely deprived of all thyroid tissue by (1) histological examination of *all* serial sections of *all* tissues from the base of the tongue to and including the trachea, esophagus, thymus, heart, pericardium, and associated great vessels and (2) the absence of iodine-concentrating tissues in the neck and mediastinal regions as measured by radioautographic procedures.

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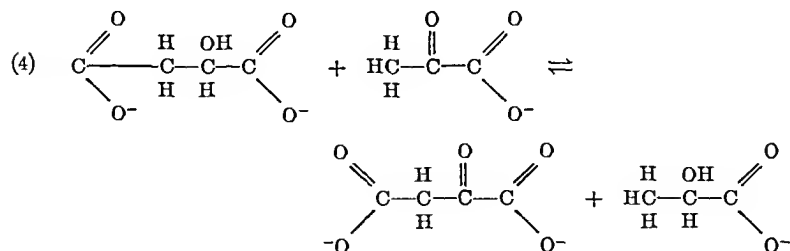
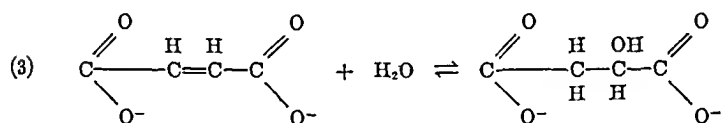
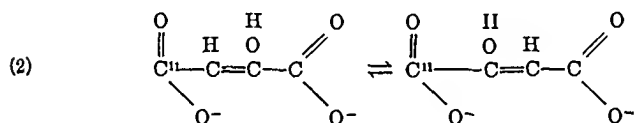
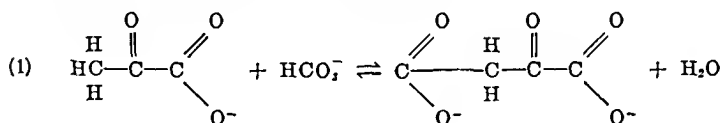
# THE MECHANISM OF CARBON DIOXIDE FIXATION IN CELL-FREE EXTRACTS OF PIGEON LIVER\*

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The fixation of  $C^{14}O_2$  by cell-free extracts of pigeon liver has been reported previously (1). This paper deals with a detailed study of these extracts and describes a preparation in which the initial fixation and the subsequent distribution of labeled carbon are apparently the result of the occurrence of the accompanying reversible reactions.



Reactions 1 and 2 occur when pyruvate alone is present as a substrate. Reactions 3 and 4 occur in addition when fumarate or malate is added with pyruvate.

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### Methods

Pyruvate was determined by the carboxylase method (2), succinate according to Krebs' modification (3) of Szent-Györgyi and Gözsy's procedure (4), fumarate and malate according to Krebs, Smyth, and Evans (5),  $\alpha$ -ketoglutarate according to Krebs and Eggleston (6), citrate according to Pucher, Sherman, and Vickery (7), lactate according to Friedemann and Graesser (8). Total carbon dioxide was determined by pipetting an aliquot into 10 per cent NaOH in a Wurburg vessel and measuring, manometrically, the carbon dioxide liberated on tipping acid in from the side arm. In all those experiments in which analyses were made for total carbon dioxide, incubation was carried out in a vessel completely filled with the reaction mixture and tightly stoppered so that there was no gas phase present.

Suitable dilutions of redistilled standard molar pyruvic acid were neutralized immediately before use with 1 M  $\text{NaHCO}_3$ . Oxalacetic acid was prepared from the sodium salt of diethyl oxalacetate according to Krampitz and Werkman (9). All acidic substances were added as the neutral sodium salts.

*Preparation of Enzyme*—The livers were removed from pigeons immediately after decapitation, cooled on ice, and minced. The mince was immediately suspended in 5 volumes of ice-cold acetone, stirred for 5 minutes, and filtered off with suction. If the powder thus obtained did not dry rapidly, the acetone treatment was repeated. Such powders, stored in a vacuum desiccator in the refrigerator, were stable for a month or longer. The crude enzyme mixture was prepared by extracting the acetone powder for 5 minutes at  $40^\circ$  with 8 volumes of distilled water and centrifuging off the solid particles. The red-brown supernatant was clear or cloudy, depending apparently on the glycogen content of the livers used. This extract was either used directly or was dialyzed or precipitated with ammonium sulfate. Further details are given in the description of the individual experiments.

*Assay for Utilization of  $\text{C}^{14}\text{O}_2$* —Carbon dioxide utilization was measured by incubating a given preparation at  $40^\circ$  with various additions in the presence of a known amount of  $\text{C}^{14}\text{O}_2$  (added as an aliquot of a dilute  $\text{Na}_2\text{C}^{14}\text{O}_3$  solution). At the end of the reaction time the mixture was deproteinized with a measured amount of 10 per cent metaphosphoric acid and filtered.  $\text{C}^{14}\text{O}_2$  was removed from the filtrate by bubbling carbon dioxide through the solution for 30 minutes. Control experiments in which  $\text{C}^{14}\text{O}_2$  was added to filtrates after precipitation with metaphosphoric acid showed that this treatment adequately removed all traces of labeled carbon present as carbon dioxide. The radioactivity remaining in the filtrate, therefore, represented the total amount of  $\text{C}^{14}$  fixed in organic form. The radioactivity of aliquots of the filtrate and of the original  $\text{Na}_2\text{C}^{14}\text{O}_3$  solution was

measured quantitatively with a Geiger-Müller counter. The  $C^{11}$  fixation was calculated and expressed as per cent of the total  $C^{11}$  initially added to the sample. The amount of carbon represented by a given amount of radioactivity will vary with the total amount of available carbon in which the  $C^{11}$  may be distributed. This was not the same in different experiments, since a variety of procedures was employed. While at first the enzyme was assayed under conditions similar to those employed in working with fresh liver mince (10), nitrogen was substituted for oxygen when it was found that fixation occurred just as readily under anaerobic as under aerobic conditions. In the experiments described in Tables I and III, 4 ml. of Ca-free Krebs' bicarbonate-saline plus the various substrates were equilibrated with 95 per cent  $N_2$ , 5 per cent carbon dioxide in 50 ml. vessels at  $40^\circ$ . Then 4 ml. of enzyme and 1 ml. of  $Na_2C^{11}O_3$  were added, the vessels were stoppered, and the mixture was incubated with shaking for 20

TABLE I

*Effect of Substrates on Fixation of  $C^{11}O_2$  by Crude Extracts*

Each vessel contained 4 ml. of acetone powder extract, 4 ml. of Ca-free Krebs' bicarbonate-saline, 1 ml. of  $Na_2C^{11}O_3$  solution plus substrates as indicated; incubated 20 minutes at  $40^\circ$  in an atmosphere of 5 per cent  $CO_2$ , 95 per cent  $N_2$ .

Substrate	$C^{11}$ fixed (as per cent of total $C^{11}$ added)	
	Experiment I	Experiment II
None.....	3.3	2.7
0.5 ml. 0.5 M pyruvate.....	21.1	15.9
0.5 " 0.5 " fumarate.....	4.7	5.6
0.5 " 0.5 " pyruvate + 0.5 ml. 0.5 M fumarate.	36.3	44.1

minutes. Later it was found that the bicarbonate-saline could be omitted and incubation was carried out in small test-tubes without shaking. A series of twenty such tubes could easily be set up in one experiment for assay with one preparation of radioactive carbon.

*Utilization of  $C^{11}O_2$  by Undialyzed Enzyme Extracts*—Undialyzed enzyme extracts prepared as described above utilized small amounts of  $C^{11}O_2$  when incubated without added substrates. The addition of pyruvate caused an increase in the quantity of  $C^{11}O_2$  fixed. While the addition of fumarate alone had little effect, the addition of both fumarate and pyruvate gave a further increase in fixation. These effects are illustrated in Table I which gives results obtained with two separate preparations.

*Properties of Precipitated and Dialyzed Extracts*—The enzyme system responsible for carbon dioxide utilization could be precipitated by two-thirds saturation with ammonium sulfate. After resuspension of the pre-

precipitate in water, and dialysis against phosphate buffer, the necessity of pyruvate, fumarate, and a boiled tissue extract for a large  $C^{14}$  uptake could be very clearly demonstrated. A typical experiment is shown in Table II.

The fact that dialysis and precipitation by ammonium sulfate resulted in little loss of activity is shown in Table III. Dialysis against 0.025 M phosphate, pH 7.4, resulted in preparations with somewhat higher activity than those obtained by dialysis against distilled water. In each case the effect of pyruvate, fumarate, and boiled tissue extract was demonstrated.

While the nature of the activating substances in the boiled tissue extracts is not known with certainty, the substitution of  $Mn^{++}$  and a crude cozymase

TABLE II

*Effect of Tissue Extract and Substrates on  $C^{14}O_2$  Fixation by Ammonium Sulfate Precipitates*

48 ml. of an  $H_2O$  extract of the acetone powder of pigeon liver + 24 ml. of saturated  $(NH_4)_2SO_4$  were used. The precipitate was centrifuged off and discarded. 34 ml. of saturated  $(NH_4)_2SO_4$  were added to 67 ml. of supernatant. The precipitate was resuspended in  $H_2O$  and dialyzed for 24 hours at  $0^\circ$  against 0.025 M phosphate, pH 7.4. The final enzyme volume was 15 ml. Incubation was carried out in stoppered test-tubes at  $40^\circ$  for 30 minutes. Each tube contained 0.5 ml. of 0.154 M  $NaHCO_3$  saturated with  $CO_2$  and 0.5 ml. of  $Na_2C^{14}O_3$  solution plus other additions as indicated.

Additions				$C^{14}$ fixed (as per cent of total $C^{14}$ added)
Enzyme	Tissue extract*	0.5 M sodium pyruvate	0.5 M sodium fumarate	
ml.	ml.	ml.	ml.	
	2	0.2	0.2	0.0
2		0.2	0.2	0.2
2	2		0.2	1.6
2	2	0.2		1.4
2	2	0.2	0.2	32.8

\* 1 part of freshly ground beef liver and 1 part of water heated for 5 minutes in a boiling water bath, and filtered.

mase preparation for such extracts (boiled muscle extracts were as effective as boiled liver extracts) gave mixtures almost as active as those obtained with the extracts themselves (Table IV).

The identity of manganese and cozymase with the activating agents present in boiled tissue extracts is, of course, not proved by such an experiment, particularly in view of the relatively large quantity of crude cozymase employed. It seems probable, however, that at least part of the effect of boiled tissue extracts is due to the presence of these substances.

*Chemical Reactions Occurring during Carbon Dioxide Utilization. Evidence for Reactions 1 and 2: Enzymic Decarboxylation of Oxalacetic Acid—*

When pyruvate alone, without added fumarate, is incubated with bicarbonate in the presence of dialyzed enzyme supplemented with manganese

TABLE III

*Comparison of  $C^{14}O_2$  Fixation by Various Preparations*

Enzyme 1, original undialyzed extract prepared as in Table II; Enzyme 2, prepared by dialyzing Enzyme 1 against distilled water; Enzyme 3, prepared by dialyzing Enzyme 1 against 0.025 M phosphate, pH 7.4; Enzyme 4, prepared by precipitating 30 ml. of Enzyme 1 at two-thirds saturation with ammonium sulfate, redissolving in water, and dialyzing against 0.025 M phosphate, pH 7.4; final volume 24 ml. All dialyses were carried out for 24 hours at ice box temperature. Each vessel contained 4 ml. of Ca-free Krebs' bicarbonate-saline, 1 ml. of  $Na_2C^{14}O_3$ , plus additions as indicated; incubated 20 minutes at 40° in an atmosphere of 5 per cent  $CO_2$ , 95 per cent  $N_2$ .

Enzyme No.	Additions				$C^{14}$ fixed (as per cent of total $C^{14}$ added)
	Amount of enzyme	Tissue extract*	0.5 M sodium pyruvate	0.5 M sodium fumarate	
	ml.	ml.	ml.	ml.	
1	4		0.5		13.9
2	4		0.5		0.6
2	2	2	0.5		4.8
2	2	2	0.5	0.5	14.9
3	4		0.5		1.8
3	2	2	0.5		5.5
3	2	2	0.5	0.5	23.1
4	4		0.5		0.3
4	2	2	0.5		5.3
4	2	2	0.5	0.5	22.3

\* Prepared by heating Enzyme 1 for 5 minutes in a boiling water bath and filtering.

TABLE IV

*Comparison between Effect of Tissue Extract and Other Additions*

2.0 ml. of enzyme (dialyzed against 0.025 M phosphate, pH 7.4), 0.5 ml. of 0.154 M  $NaHCO_3$  saturated with  $CO_2$ , 0.2 ml. of 0.5 M pyruvate, 0.2 ml. of 0.5 M fumarate, and 0.5 ml. of  $Na_2C^{14}O_3$  solution; incubated 1 hour at 40°.

	Per cent $C^{14}$ fixed
Enzyme + 2 ml. boiled muscle extract.....	43
" + 0.6 ml. 0.005 M $MnCl_2$ + 0.4 ml. cozymase solution*.	32

\* Prepared according to Williamson and Green (11), 1 mg. per ml.

and cozymase, no chemical change can be detected in the reaction mixture (Table V). Under these conditions a small amount of  $C^{14}$  is fixed, however, and practically all this radioactivity appears in the pyruvate. Thus,

in a separate experiment similar to that in Table V, with pyruvate incubated 20 minutes in the presence of  $\text{C}^{14}\text{O}_2$ , 0.7 per cent of the added  $\text{C}^{14}$  was fixed in 20 minutes. The addition of excess dinitrophenylhydrazine precipitated 96 per cent of the fixed radioactivity. The supernatant remaining after the removal of the radioactive precipitate contained 12 per cent of the fixed  $\text{C}^{14}$ . Although the recovery, 108 per cent, is higher than theoretical, the deviation is less than the sum of the possible errors, and the experiment demonstrates that under these conditions the pyruvate contains practically the whole of the utilized carbon dioxide.

Apparently an exchange reaction has occurred whereby the pyruvic acid added to the solution has become radioactive. The simplest explanation for this effect is to postulate that there is in the enzyme solution an equilibrium represented by Reactions 1 and 2. This assumes a reversible shift in the position of the hydroxyl group of the enol form of oxalacetic acid.

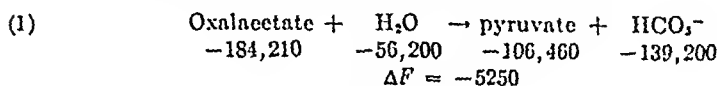
TABLE V  
*Action of Enzyme on Pyruvate Alone*

10 ml. of enzyme dialyzed against 0.025 M phosphate, pH 7.4, 3 ml. of 0.005 M  $\text{MnCl}_2$ , 2 ml. of coenzyme (1 mg. per ml.), 2 ml. of 0.15 N  $\text{NaHCO}_3$  saturated with  $\text{CO}_2$ , 1 ml. of pyruvate; incubated 1 hour at 39°.

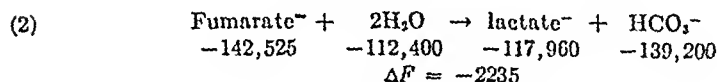
	microliters
Initial $\text{CO}_2$ .....	843
Final $\text{CO}_2$ .....	840
Initial pyruvate.....	976
Final pyruvate.....	941

Calculation<sup>1</sup> of the equilibrium point of Reaction 1 shows it to be far to the left. Our present analytical methods are not sufficiently delicate to

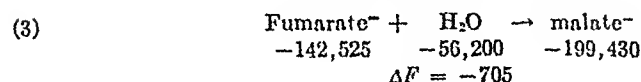
<sup>1</sup> The equilibrium constants for the reactions discussed here can be calculated from the values for the free energy of formation at 38°.



$$\frac{(\text{Pyruvate}^-)(\text{HCO}_3^-)}{(\text{Oxalacetate})} = 4.92 \times 10^3$$



$$\frac{(\text{Lactate}^-)(\text{HCO}_3^-)}{(\text{Fumarate})} = 37$$



show the presence of the small amount of oxalacetate which should be present. However, we have been able to demonstrate in our preparations an active thermolabile enzyme catalyzing the reverse reaction; namely, the decarboxylation of oxalacetic acid to pyruvate and carbon dioxide.

This enzyme is active under the conditions employed for the  $C^{14}$  utilization experiments, but it can be more easily demonstrated and studied at a pH sufficiently acid to allow the direct manometric measurement of the liberated carbon dioxide. A sodium acetate-acetic acid buffer mixture of pH 5 was found to be suitable.

Fig. 1 shows experiments which demonstrate the presence of the enzyme and the fact that it can be activated by divalent manganese ions. The enzyme alone, without addition of manganese, shows some activity in spite of the prolonged dialysis. There is a small spontaneous decarboxylation without added enzyme or manganese, and a slight catalytic effect is shown by the heated enzyme, but the rates are much lower than those observed with the unheated enzyme plus manganese. The addition of diphosphothiamine<sup>2</sup> had no effect on the rate of decarboxylation. The possibility that the enzyme may contain bound diphosphothiamine has not yet been ruled out.

The sensitivity of the enzyme to heat treatment is shown in Fig. 2. The data here clearly indicate that the catalytic effect observed differs from that caused by the thermostable substance described by Breusch (12).

The enzyme, which we shall call oxalacetate carboxylase, seems to be similar to the bacterial enzyme described by Krampitz and Werkman (9), but differs in at least one important respect; it is not appreciably activated by magnesium ions. The comparative effects of similar concentrations of manganese and magnesium ions are shown in Fig. 3.

Oxalacetate carboxylase converts oxalacetic acid quantitatively to carbon dioxide and pyruvate. The amount of carbon dioxide obtained from a given oxalacetic acid solution when the enzyme reaction has gone to com-

$$\frac{(\text{Malate}^-)}{(\text{Fumarate}^-)} \approx 3.2$$

The relationship at equilibrium between lactate, bicarbonate, fumarate, and malate can better be visualized by combining (2) and (3) to give

$$\frac{(\text{Lactate})(\text{HCO}_3^-)}{(\text{Fumarate} + \text{malate})} = 37/4.2 = 9$$

especially since analyses are listed for the sum of fumarate + malate. The free energy values used in these calculations are from an unpublished manuscript of Professor H. Borsook of the California Institute of Technology. We are indebted to Dr. Borsook for permission to use them.

<sup>2</sup> We are grateful to Merck and Company, Inc., for the gift of this material.

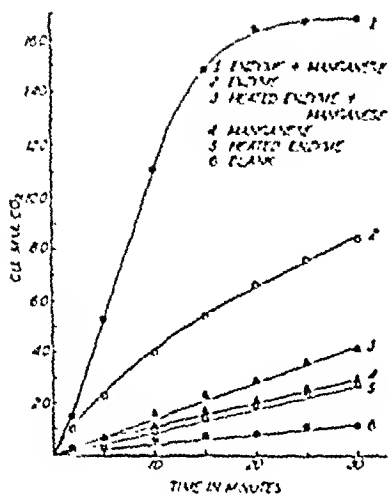


FIG. 1

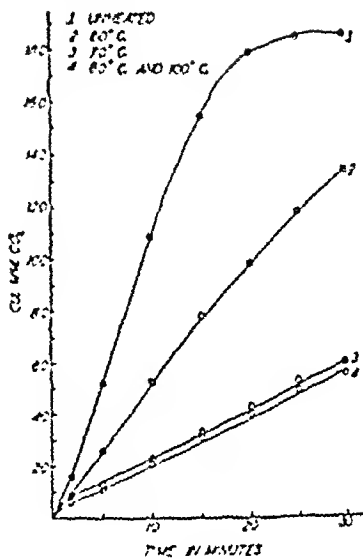


FIG. 2

FIG. 1. Demonstration of a heat-labile enzyme which decarboxylates oxalacetic acid. Each vessel contained 0.5 ml. of 0.1 M acetate buffer, pH 5.0, and water to make a final volume of 2.0 ml. Manganese was added as 0.1 ml. of 0.01 M  $\text{MnCl}_2$ ; enzyme, 0.5 ml. of dialyzed acetone powder extract of pigeon liver; 0.1 ml. of oxalacetic acid was tipped from the side arm at 0 time after equilibration at 25°. The heated enzyme was held in a boiling water bath 1 minute.

FIG. 2. The heat inactivation of oxalacetate carboxylase. Each vessel contained 0.3 ml. of enzyme held in a water bath for 1 minute at the temperatures indicated, 0.5 ml. of 0.1 M acetate buffer, pH 5.0, 0.1 ml. of 0.01 M  $\text{MnCl}_2$ , and water to make a final volume of 2 ml.; 0.1 ml. of oxalacetic acid was tipped from the side arm at 0 time after equilibration at 25°.

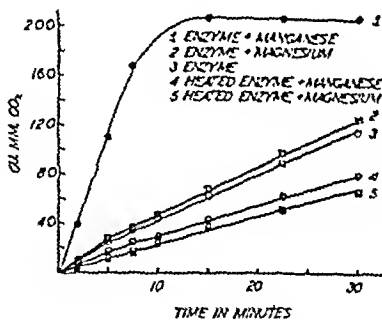


FIG. 3. Comparison of manganese and magnesium effects on oxalacetate carboxylase. Each vessel contained 0.2 ml. of enzyme, 0.5 ml. of 0.1 M acetate, and water to make a final volume of 2.0 ml.; Mn and Mg were added as 0.1 ml. of 0.01 M chloride; 0.1 ml. of oxalacetic acid was tipped from the side arm at 0 time after equilibration at 25°.

pletion is identical with that obtained by simultaneous decarboxylation of an aliquot of the same solution with aniline citrate, under the conditions of Edson (13). The pyruvate formed, when determined by the carboxylase method, is equal to the carbon dioxide evolved, after correction is made for the spontaneous decarboxylation which occurs before the oxalacetic acid is tipped into the enzyme system. There is no appreciable evolution of carbon dioxide from  $\alpha$ -ketoglutaric acid or acetoacetic acid when these compounds are added to solutions of the enzyme.

The enzyme is completely inhibited by 0.01 M malonate under the conditions described in Fig. 1.

We have been unable as yet to find any oxalacetate carboxylase in dialyzed extracts of acetone powders from the livers of other species. When the simple procedure used to make and test the pigeon liver preparations was applied to rabbit, rat, and beef liver, there was no evidence for the existence, in these preparations, of a thermolabile catalytic agent. This is in accord with the fact that, while the livers of these species utilize  $C^{14}O_2$  when used as fresh suspensions, we have not succeeded, as yet, in preparing active cell-free extracts from them. When dialyzed extracts of acetone powders prepared from rabbit muscle and pigeon breast muscle were examined, however, some thermolabile enzyme activity could be demonstrated, though it was less than one-tenth the usual amount of activity obtained from an equal weight of the acetone powder of pigeon liver.

The activity of the crude pigeon liver preparation is shown by an approximate  $Q_{CO_2}$  calculation. In the experiment shown in Fig. 3, the complete system evolved carbon dioxide at a rate of 19 microliters per minute (after correction for the carbon dioxide evolution of heated enzyme and manganese). The amount of extract used was 0.2 ml., containing about 2 mg. of dry weight. The  $Q_{CO_2}$  was therefore  $19 \times 60/2 = 570$  microliters per mg. per hour.

These facts, then—the appearance of radioactivity in pyruvate added to the dialyzed enzyme and the demonstration of an enzyme catalyzing the decarboxylation of oxalacetate—constitute evidence for the occurrence of Reactions 1 and 2.

*Evidence for Reactions 3 and 4*—The enzyme mixture contains an active fumarase. The equilibrium between malate and fumarate is approached rapidly from both sides. When 4 ml. of dialyzed enzyme were incubated with 2 ml. of 0.1 M fumarate, the ratio, fumarate added to fumarate recovered, was 4.16 after 5 minutes incubation, and 4.29 after 10 minutes. These values are in agreement with the average of 4.17 reported by Krebs, Smyth, and Evans (5) for pigeon breast muscle at 40°. Further, malate has an effect similar to that of fumarate on the utilization of  $C^{14}O_2$ .

The chemical changes that occurred when fumarate and pyruvate were

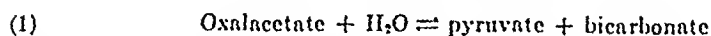
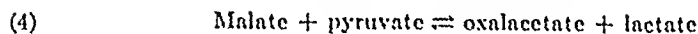
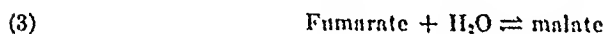


added simultaneously to a dialyzed enzyme preparation supplemented with manganese and cozymase are shown in Table VI. Within the limits of error of the procedure, there was no change in the concentration of pyruvate. For every mole of fumarate (fumarate and malate) disappearing, a mole of lactic acid and a mole of carbon dioxide were formed. No succinate, citrate,<sup>3</sup> or  $\alpha$ -ketoglutarate formation could be detected. The changes listed in Table VI constitute a complete carbon balance and must therefore represent the net result of all the chemical changes occurring during the carbon dioxide utilization. The over-all reaction is fumarate +  $2\text{H}_2\text{O} \rightarrow$  lactate +  $\text{HCO}_3^-$ , which can be subdivided into the following reactions.

TABLE VI  
*Chemical Changes Occurring during  $\text{C}^{14}\text{O}_2$  Fixation*

The enzyme was prepared by dialysis for 3 days against 0.025 M phosphate buffer, 10 ml. of enzyme, 3.0 ml. of 0.005 M  $\text{MnCl}_2$ , 2 ml. of 0.1 per cent crude cozymase preparation, 2 ml. of 0.154 N  $\text{NaHCO}_3$  saturated with  $\text{CO}_2$ , 11,200 microliters of pyruvate in 1 ml., and 9400 microliters of fumarate in 1 ml.; incubated 12 hours at  $39^\circ$ . No gas phase.

	Pyruvate	Lactate	Total $\text{CO}_2$	Fumarate + malate
	microliters	microliters	microliters	microliters
Initial.....	11,400	130	8,970	9400
Final.....	11,200	4330	13,180	4980
$\Delta$ .....	-200	+4200	+4,210	-4420



There may be other intermediary steps but they do not lead to the accumulation of intermediates which can be distinguished from the initial and final reactants by the analytical procedures employed. It should be noted that the small amount of oxalacetate which might accumulate would be determined in the analyses as pyruvate and carbon dioxide. Application of Straub's (14) color test for oxalacetate to the reaction mixture gave a faint positive reaction. As one would expect, this test was much stronger when carried out on an enzyme-substrate incubation mixture containing cozymase but no manganese.

The utilization of carbon dioxide by these dialyzed extracts in the presence of fumarate, pyruvate, and the necessary cofactors must be asso-

<sup>3</sup> We are obliged to Dr. Joseph J. Ceithaml for the citrate analyses.

ciated with Reactions 1 to 4 and can be explained in terms of their reversibility. The radioactive carbon taken up in the oxalacetate (Reaction 1) becomes distributed in the fumarate, malate, and lactate through the reversibility of Reactions 3 and 4 and in the pyruvate by Reaction 2. The increased quantity of carbon dioxide utilized when fumarate is added with pyruvate can be explained, then, by the accumulation of radioactive carbon in the carboxyl groups of these various monocarboxylic and dicarboxylic acids. Support for this view is furnished by an experiment set up as in Table VI, but with  $C^{14}$  added. 68 per cent of the  $C^{14}$  was fixed. In contrast to the experiment previously described in which pyruvate alone was used as a substrate, only 15 per cent of the fixed carbon dioxide was found in the pyruvate; that is, was precipitated by dinitrophenylhydrazine. The remaining 85 per cent was found in the filtrate and in view of the lack of other soluble intermediates must exist as lactate, malate, and fumarate.

The carbon dioxide formation which occurs in the presence of pyruvate and fumarate (Reactions 4 and 1) can be followed manometrically when the reaction occurs at pH 5.5 in an acetate buffer, under conditions similar to those employed in Fig. 1. The procedure has advantages over more laborious chemical analyses and has been used extensively to study the effects of cofactors and inhibitors and the substitution of other substrates for pyruvate and fumarate.

#### DISCUSSION

The utilization of  $CO_2$  by heterotrophic bacteria was first demonstrated by Wood and Werkman (15).

By the use of radioactive  $C^{14}O_2$  it has been possible to demonstrate a direct utilization of carbon dioxide by animal tissues in the synthesis of  $\alpha$ -ketoglutaric acid from pyruvic acid in pigeon liver (10).

Early work in Krebs' laboratory on the utilization of pyruvate in pigeon liver (16), the work of Krebs and his collaborators (17), the work of Wood and Werkman on propionic acid bacteria (18), and their later investigations on minced pigeon liver (19) suggested very strongly that the initial step in this utilization of carbon dioxide was the formation of oxalacetic acid. However, in view of the complexity of the systems studied in these investigations it seemed desirable to study these reactions with simplified enzyme systems, particularly so since the radioactive carbon in the synthesized  $\alpha$ -ketoglutaric acid represented only a small fraction of the total quantity of carbon dioxide fixed.

The work presented in this paper deals with a system so simplified that the chemical reactions are few in number and are, in their net terms, completely described. It is possible to demonstrate the carboxylation of pyru-

vate in a system uncomplicated by the simultaneous occurrence of the more controversial phases of the citrate cycle. The enzyme responsible for the decarboxylation of oxalacetate is most probably the enzyme involved in the carboxylation itself and is similar to, although apparently not identical with, the one found in bacterial preparations by Krampitz and Werkman (9).

Perhaps the most uncertain feature of the conclusions drawn is the shift in the enol form of the oxalacetate (Reaction 2) first postulated by Meyerhof (20). This reaction is hypothetical and is included with the other reactions, for all of which good evidence is available, because it is the simplest explanation for the incorporation of labeled carbon in the pyruvate molecule when pyruvate alone is added as a substrate.

It would be possible to explain the experimental facts without postulating this reaction if it was assumed that the enzyme preparation contained a quantity of lactate, or some other metabolite, sufficient to reduce the oxalacetic acid formed from carbon dioxide and pyruvate to malate which would be in equilibrium with the symmetrical fumarate molecule. Since the amount of reductant required would be very small, this possibility cannot be excluded.

The omission of phosphate from the formulae does not imply a belief that phosphate may not be involved. All the enzyme preparations used contained phosphate. There was no evidence for the formation of organic phosphates during the course of the reaction, but no attempt has yet been made to ascertain the effect on the reactions of the complete removal of phosphate.

It is of interest to compare the carboxylation of pyruvate in animal tissues with the photosynthetic utilization of carbon dioxide in plants that represents the ultimate source of carbon for organic compounds. This latter process can be regarded as involving a preliminary non-photosynthetic, so called, "dark reaction," whereby carbon dioxide is converted into the carboxyl group of some organic compound followed by a series of photosynthetic reactions leading to the ultimate formation of carbohydrate (21). There is, then, a net uptake and reduction of carbon dioxide during this process. In liver, the utilization of carbon dioxide also involves, as we have seen, an initial carboxylation, although the products of the reaction in plant and animal are apparently quite different. The subsequent fate of the initial product of fixation in the animal will determine to what extent we can regard the carbon dioxide fixation as involving a reduction of carbon dioxide. The appearance of large quantities of radioactive carbon in a compound does not indicate that a net uptake of carbon dioxide is involved. For example, when the dicarboxylic acids are converted into glycogen, the reaction, according to our present information, probably in-

volves the conversion of the acid into a 3-carbon particle with the loss of a molecule of carbon dioxide, so that no net uptake of carbon dioxide occurs (22, 23). Our information about the mechanisms by which fat is synthesized is slight, but if a 3-carbon carbohydrate particle is a necessary intermediate in the synthesis of fat from the dicarboxylic acids, then neither the formation nor storage of carbohydrate or fat would result in a net uptake of carbon dioxide in the animal organism. However, to the extent that fat may be synthesized and stored from the dicarboxylic acids or from the 2-carbon particles resulting from the splitting of the dicarboxylic acid, or to the extent that there is a direct conversion of the dicarboxylic acid into protein material, and its storage as such, for example by the formation of aspartic acid, this would represent a mechanism whereby the animal would convert carbon dioxide into tissue constituents with a net uptake of carbon dioxide.

#### SUMMARY

It is possible to prepare cell-free pigeon liver extracts containing the enzyme system involved in the initial reactions of carbon dioxide fixation. These extracts contain a heat-sensitive enzyme, activated by Mn ions, catalyzing the decarboxylation of oxalacetic acid. This oxalacetate carboxylase is also involved, presumably, in the carboxylation reaction.

The aqueous extracts containing this enzyme represent a system so simplified that the chemical reactions which occur (represented by Reactions 1 to 4) can be, in their net terms, completely described. It is concluded that the initial reaction involved in carbon dioxide fixation by pigeon liver is the Wood and Werkman reaction; *i.e.*, the condensation of carbon dioxide and pyruvic acid to oxalacetic acid.

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# A SPECIFIC ENZYMATIC METHOD FOR THE DETERMINATION OF NICOTINIC ACID IN BLOOD\*

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There are numerous chemical methods for the determination of nicotinic acid in blood (1-12). These are based on the colorimetric reaction of pyridine derivatives with cyanogen bromide and an aromatic amine such as aniline, *p*-aminoacetophenone, or *p*-methylaminophenol sulfate (elon or metol), first suggested by Bandier and Hald (13).

The reaction between nicotinic acid, cyanogen bromide, and an aromatic amine is not highly specific, since there are other pyridine derivatives which also produce color with cyanogen bromide and aromatic amines. This difficulty has been partially overcome in the methods with cyanogen bromide and aminophenol by the introduction of potassium dihydrogen phosphate, and the use of Lloyd's reagent. These modifications do not yield specific results, because the cyanogen bromide-aminophenol reagent even in the presence of potassium dihydrogen phosphate still reacts with nicotinamide, nicotinuric acid, diethyl nicotinamide, nicotine, and other substances to a lesser degree. Moreover, Lloyd's reagent adsorbs a great variety of substances which might influence the color reaction.

In the method described the factor of inadequate specificity and the process of adsorption on Lloyd's reagent are eliminated. It is a colorimetric procedure which is rendered specific by the use of bacterial enzymes. The bacteria are grown on a synthetic<sup>1</sup> medium containing nicotinic acid as their sole source of carbon and nitrogen. They produce adaptive enzymes which are contained in the washed bacteria and which may then be used to decompose nicotinic acid. This concept of the production of adaptive enzymes was expounded by Karström (14), Dubos and Miller (15), and Dubos (16). This technique has been successfully utilized in the determination of creatine and creatinine in blood (unpublished data of the author), in the analysis of creatinine in tissues (17, 18), and in the determination of creatinine in body fluids (19). In the case of the creatine-creatinine-decomposing enzymes, a soluble extract has been prepared from the desiccated washed cells (20).

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### Method

The method is based on the colorimetric reaction between nicotinic acid, clon, and cyanogen bromide, adapted from Bandier and Hald (13) and Perlzweig *et al.* (21). Simultaneous determinations are carried out on samples of blood filtrates incubated with heated bacterial cells to obtain the total chromogenic material estimated as total nicotinic acid (Reading 1) and on separate aliquots incubated with unheated bacteria to obtain residual chromogen estimated as residual nicotinic acid (Reading 2). The difference between Reading 1 and Reading 2 represents "true nicotinic acid."

#### Reagents—

10 per cent  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  solution.

0.07 N HCl solution.

0.4 M  $\text{KH}_2\text{PO}_4$  solution.

2.5 N  $\text{H}_3\text{PO}_4$  solution.

CNBr reagent. This is prepared by the careful addition of cold saturated bromine water to a cold freshly prepared solution of 10 per cent NaCN until the yellow color disappears. This solution is stable for over 3 weeks at 5°. The pH of the CNBr solution is adjusted to 4.5 with HCl prior to its use.

*p*-Methylaminophenol sulfate, solid, Eastman Kodak clon.

Nicotinic acid standard. This contains 10  $\gamma$  per cc. in water, freshly prepared from a stock solution of 2 mg. per cc. of nicotinic acid in absolute alcohol.

Bacterial suspension (heated and unheated portions).

*Preparation of Bacterial Cultures*—The microorganism used is the NC (neutral culture) isolated from soil by Dubos and Miller (15). The author has kept this culture since 1939. It is kept on plain agar. The organisms are transferred from a 24 hour agar slant to tryptone broth prepared as described previously (17). A loopful of a 24 to 48 hour tryptone culture is inoculated into a 125 cc. Erlenmeyer flask containing 25 cc. of the medium. The medium contains 3 gm. of nicotinic acid, neutralized to pH 7.0 with sodium hydroxide, 5 gm. of sodium chloride, 25 cc. of M phosphate buffer, pH 7.0, made according to Green (22), 200 mg. of Difco Bacto-yeast extract, and "artificial tap water" (17) up to 1 liter. Transfers are made to the same medium after 30 hours (it is occasionally necessary to incubate the first transfer as long as 72 hours to get heavy growth). When the organism is adapted by several transfers, the entire contents of each 125 cc. flask are inoculated into a 4 liter Erlenmeyer flask containing 500 cc. of medium, and incubated for 30 hours at 37.5°. The bacteria are separated by centrifugation, washed three or four times to remove all nicotinic acid,

and resuspended in a volume of sterile water equal to one-fortieth of the original culture medium. The activity of the culture is maintained by weekly transfers to the same medium. An interesting characteristic of this organism is the formation of green pigment in old cultures or at low temperatures.

*Method of Assay of Bacterial Suspension*—50 cc. unstoppered Lusteroid centrifuge tubes are used. Reagent blanks, blanks containing reagents plus bacteria, tubes with 10  $\gamma$  of standard solutions of nicotinic acid, and assays containing 20 and 40  $\gamma$  of nicotinic acid plus amounts of bacterial suspension varying from 0.05 to 0.5 cc. are set up simultaneously. All tubes receive 0.3 cc. of 0.5  $M$  phosphate buffer, pH 7.0, and water to 5.5 cc. These are incubated at 37.5° for 30 minutes, then centrifuged at high speed for 20 minutes, and the clear supernatant fluid poured off into test-tubes. 4.0 cc. aliquots are pipetted into colorimeter test-tubes; the pH is brought to 4.5 with 0.10 to 0.11 cc. of 2.5  $N$  phosphoric acid (with brom-cresol green as an external indicator). The amount of acid required is readily determined on the residual solutions after the 4.0 cc. aliquots have been removed. All solutions are then analyzed as in the procedure for blood filtrates (see below). By such an assay it is found that 1 cc. of bacterial suspension decomposes or renders non-chromogenic 200  $\gamma$  or more of nicotinic acid in 30 minutes. The cells retain their activity for at least 1 month at 5°. If desired, the bacteria may be desiccated *in vacuo* over phosphorus pentoxide; a more stable product is obtained thus but with the loss of two-thirds of the activity of the washed cells.

*Specificity of Bacterial Enzymes*—The activity of the enzymes present in the washed bacterial cells was tested on nicotinic acid and various derivatives by incubation of these substances with heated and unheated bacteria for 30 minutes at 37.5°. The procedure was similar to that used in blood filtrates (see below). Since the chromogenic value of the derivatives was much less than that of nicotinic acid, it was necessary to use much larger amounts of these substances. Sufficient bacterial suspension was used to decompose or render non-chromogenic at least 20  $\gamma$  of nicotinic acid. Table I reveals that the bacterial enzymes did not appreciably affect the chromogenic property of *freshly prepared solutions* of nicotinamide, diethyl nicotinamide, or nicotinuric acid. This is of great significance in the case of nicotinamide which gives one-fourth of the color of nicotinic acid. The persistence of its color reaction after incubation with the washed bacteria illustrates the marked degree of specificity of these enzymes. It might also be noted that the same bacteria when grown on a medium containing creatinine as the sole source of carbon and nitrogen did not produce enzymes which attacked nicotinic acid.

*Determination of True Nicotinic Acid in Blood Filtrates*—More constant



and reproducible "true nicotinic acid" values were obtained on acid-hydrolyzed tungstic acid blood filtrates than on blood samples which were directly hydrolyzed before removal of the proteins. Hence this method was used.

1:5 tungstic acid filtrates of oxalated whole blood are prepared. The precipitated protein is separated by centrifugation and the supernatant fluid is recentrifuged to eliminate the small particles. 15 cc. of clear filtrate are then treated with 3 cc. of 8 N hydrochloric acid for at least 1 hour in a water bath at 90-95°. A greenish precipitate forms which disappears on neutralization. The solution is approximately neutralized with 8 N sodium hydroxide in the presence of 0.01 cc. of phenolphthalein, and then brought to pH 7.0 with 1 N hydrochloric acid (in the presence of brom-thymol blue as external indicator). 1.4 cc. of 0.5 M phosphate buffer, pH 7.0, are now added to the solution which is clear and colorless and has a volume

TABLE I

*Specificity of Nicotinic Acid-Decomposing Bacterial Enzymes for Nicotinic Acid and Derivatives*

Substance	Chromogenic equivalent of nicotinic acid or derivative	Amount used	Decomposition in 30 min. at 37.5°
	<i>per cent</i>	$\gamma$	<i>per cent</i>
Nicotinic acid.....	100	10	100
Nicotinamide.....	25	40	0
Diethyl nicotinamide.....	4	100	0
Nicotinuric acid....	10	60	3

of 20 to 21 cc. To 50 cc. Lusteroid tubes are added 5.0 cc. aliquots of the blood filtrates in duplicate, a suitable quantity of bacterial suspension (which has been heated to 90° for 5 minutes to inactivate the enzymes), and water to 5.5 cc. Another series of aliquots is prepared in the same way except that unheated bacteria are added. A quantity of bacterial suspension capable of destroying at least 5 times the expected amount of total nicotinic acid (total chromogen) should be added to each of these. In addition to the above samples, reagent blanks, bacterial suspension blanks, a 10  $\gamma$  standard of nicotinic acid, and a single assay as described in the method of assay should be prepared simultaneously. The tubes are incubated and centrifuged as described above. 4.0 cc. aliquots of the clear supernatant fluid are then transferred to colorimeter tubes and analyzed as follows: The pH of each sample is adjusted to 4.5 with phosphoric acid. This adjustment of pH is important, because slight variations in acidity affect color production considerably. 0.5 cc. of 0.4 M potassium dihydro-

gen phosphate is added to each tube, and the tubes placed in a water bath at 75-80° for 5 minutes. Then, 0.5 cc. of cyanogen bromide reagent is added to each tube, the contents stirred with a glass rod, and the reaction continued in the bath for 5 minutes longer. The tubes are cooled in ice water. 150 mg. of elon are added to each tube, the contents are mixed thoroughly by inversion, and the contents are allowed to stand in the dark for 30 to 40 minutes. The percentage of light transmission of each sample is then determined in a modified Evelyn photoelectric colorimeter, the galvanometer being set at 100 with the blank which is clear and colorless. A composite filter of Corning glasses (No. 978 (one-half thickness) and No. 597 (3.93 mm.)) which has a maximum transmission at approximately 400  $m\mu$  is used. The values in percentage of light transmission are read from calibration lines prepared with pure nicotinic acid solutions. Suitable corrections are made for the dilutions of the filtrates. A final volume of

TABLE II  
*Concentration of True Nicotinic Acid in Whole Blood of Dog and Man*

Dog			Man		
Total chromogen	Residual chromogen	True nicotinic acid	Total chromogen	Residual chromogen	True nicotinic acid
$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.
4.8	0.2	4.6	4.8	1.7	3.1
4.9	0	4.9	5.3	1.3	4.0
5.3	1.0	4.3	3.4	0.3	3.1
7.2	0.6	6.6	3.3	0	3.3
8.5	1.9	6.6	5.0	1.0	4.0
5.3	1.6	3.7	4.2	1.0	3.2
			4.8	0.9	3.9
Average..6.0	0.9	5.1	4.4	0.9	3.5

5.0 cc. is used rather than the conventional 10 or 20 cc., since it was pointed out by Bandier and Hald that even if the concentration of aminophenol is increased proportionately there is twice as much color developed in 5 cc. as in 10 cc.

The values obtained on the samples which have been treated with unheated bacteria represent residual chromogen (Reading 2). These are subtracted from the values obtained on the samples which have been treated with heated bacteria and represent total chromogen (Reading 1). The difference is considered as "true nicotinic acid."

### Results

With this technique the concentration of total chromogen (total nicotinic acid) in hydrolyzed dog blood filtrates is found to be 4.8 to 8.5  $\gamma$  per cc.

(see Table II). 85 per cent of this amount is decomposed by the bacterial enzymes, indicating that this fraction is "true nicotinic acid." In like manner, human blood contains 3.3 to 5.3  $\gamma$  of total chromogen per cc.; 80 per cent of this is decomposed by the bacterial enzymes, indicating that this is the amount of "true nicotinic acid" present.

The values for total chromogen or total nicotinic acid in dog blood are in agreement with those of Pearson (5), using the cyanogen bromide-aniline method.

The values for total chromogen in human blood are in agreement with those obtained by Bandier (6), and Stotz (9), using the cyanogen bromide-aminophenol method, and with those obtained by Ritsert (3), Swaminathan (2), and Patton, Sutton, and Youmans (11), using the cyanogen bromide-aniline method.

#### SUMMARY

A specific, enzymatic, colorimetric procedure for the determination of "true nicotinic acid" in whole blood is presented. The method owes its specificity to the use of bacterial enzymes present in bacteria which have been adapted to grow on nicotinic acid.

By this method it is found that 85 per cent of the total chromogen estimated as nicotinic acid in dog blood is "true nicotinic acid," and that 80 per cent of this is "true nicotinic acid" in human blood.

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